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Synergistic Interactions of Chemotherapeutic Drugs and Tumor Necrosis Factor-related Apoptosis-inducing Ligand/Apo-2 Ligand on Apoptosis and on Regression of Breast Carcinoma *in Vivo*[1]

Thiyam Ramsing Singh, Sharmila Shankar, Xufen Chen, Mohammed Asim, and Rakesh K. Srivastava[2]

Department of Pharmaceutical Sciences, Molecular and Cellular Biology Program, Greenebaum Cancer Center, University of Maryland, Baltimore, Maryland 21201-1180

ABSTRACT

Tumor necrosis factor-related apoptosis-inducing-ligand (TRAIL/Apo-2 ligand) induces apoptosis in the majority of cancer cells without appreciable effect in normal cells. Here, we report the effects of TRAIL on apoptosis in several human breast cancer cell lines, primary memory epithelial cells, and immortalized nontransformed cell lines, and we examine whether chemotherapeutic agents augment TRAIL-induced cytotoxicity in breast cancer cells *in vitro* and *in vivo*. TRAIL induced apoptosis with different sensitivities, and the majority of cancer cell lines were resistant to TRAIL. The chemotherapeutic drugs (paclitaxel, vincristine, vinblastine, etoposide, camptothecin, and Adriamycin) induced death receptors (DRs) TRAIL receptor 1/DR4 and TRAIL receptor 2/DR5, and successive treatment with TRAIL resulted in apoptosis of both TRAIL-sensitive and -resistant cells. Actinomycin D sensitized TRAIL-resistant cells through up-regulation of caspases (caspase-3, -9, and -8). TRAIL induces apoptosis in Adriamycin-resistant MCF7 cells already expressing high levels of death receptors DR4 and DR5. The pretreatment of breast cancer cells with chemotherapeutic drugs followed by TRAIL reversed their resistance by triggering caspase-3, -9, and -8 activation. The sequential treatment of nude mice with chemotherapeutic drugs followed by TRAIL induced caspase-3 activity and apoptosis in xenografted tumors. Complete eradication of established tumors and survival of mice were achieved without detectable toxicity. Thus, the sequential administration of chemotherapeutic drugs followed by TRAIL may be used as a new therapeutic approach for cancer therapy.

INTRODUCTION

Despite early promising results, treatment of breast cancer with modulators has remained problematic. This may be explained by the fact that there is an array of alternate resistance mechanisms, controlled by different families of genes, such as those involved in apoptosis. Whereas these alternative pathways could influence drug resistance, leading to diminished cell killing by chemotherapeutic drugs, the effector molecules are poorly understood, and their relative contribution in any one disease remains to be elucidated. Therefore, there is a need to develop new anticancer drugs and novel regimens that are capable of killing drug-resistant cells. Activation of DR[3] pathway may provide a new modality in breast cancer treatment because of the ability of death receptors to directly induce apoptosis, thus bypassing cellular drug resistance. TRAIL/Apo-2 ligand suppresses growth of TRAIL-sensitive human mammary adenocarcinoma in mice and nonhuman primates without any significant toxic effects, such as those seen with tumor necrosis factor and FasL (1, 2). Although TRAIL is capable of inducing apoptosis in tumor cells of diverse origin (3–5), recent studies have shown that majority of breast cancer cell lines are resistant to the apoptotic effects of TRAIL (6), suggesting that TRAIL alone may be ineffective for breast cancer therapy. Furthermore, several studies have shown that TRAIL-resistant breast and prostate cancer cells can be sensitized by chemotherapeutic drugs *in vitro*, indicating that combination therapy may be a possibility (6–8). However, the effectiveness of the chemotherapeutic drugs and TRAIL in a human cancer xenograft model system has not been demonstrated. Therefore, understanding the molecular mechanisms of TRAIL resistance and ways to sensitize these cells to undergo apoptosis by TRAIL are important issues for effective cancer therapy.

Unlike FasL, TRAIL induces apoptosis of tumor cells but is less effective in nontransformed cells (9–12). TRAIL can bind with two DRs, TRAIL-R1 (DR4/Apo-2A; Ref. 11) and TRAIL-R2 (DR5/TRICK/Killer; Refs. 10, 13, and 14), which contain cytoplasmic death domains necessary to form functional death-inducing signaling complex and trigger apoptotic signals. Three other TRAIL-Rs, TRAIL-R3 (TRID/DcR1/LIT; Refs. 10, 13, and 15), TRAIL-R4 (TRUNDD/DcR2; Refs. 16 and 17), and osteoprotegerin (18), also bind to TRAIL. TRAIL-R3 and TRAIL-R4 have extracellular domains similar to TRAIL-R1 and TRAIL-R2 but lack a functional cytoplasmic death domain. TRAIL-R3 and TRAIL-R4 may serve as DcRs, whereas the fifth receptor, osteoprotegerin, is a secreted protein with no known membrane anchor.

Because several chemotherapeutic drugs up-regulate DRs in human cancer cells *in vitro*, it is possible that these conventional chemotherapeutic drugs might enhance the cytotoxicity of TRAIL. This approach might also be useful in killing drug-resistant cells, expressing high levels of DRs (DR4 and/or DR5), by the ligand TRAIL. The objectives of this study are (*a*) to investigate the effects of TRAIL on apoptosis in human breast normal and malignant cells and (*b*) to determine whether chemotherapeutic drugs (paclitaxel, vincristine, vinblastine, Adriamycin, etoposide, and camptothecin) augment TRAIL-induced apoptosis *in vitro* and *in vivo*. Here we demonstrate that several breast cancer cells are resistant to apoptosis by TRAIL, and chemotherapeutic drugs sensitize TRAIL-resistant cells to undergo apoptosis by up-regulating DR4 and/or DR5 and activating caspase. The chemotherapeutic drugs synergize with TRAIL in reducing tumor growth, inducing tumor cell apoptosis, and enhancing survival of tumor-bearing mice. Thus, chemotherapeutic drugs such as paclitaxel, vincristine, vinblastine, Adriamycin, etoposide, and camptothecin can be used with TRAIL to kill TRAIL-sensitive and -resistant breast cancer cells.

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[2] To whom requests for reprints should be addressed, at Department of Pharmaceutical Sciences, University of Maryland, 20 North Pine Street, Baltimore, MD 2120-1180. E-mail: rsrivast@rx.umaryland.edu.

[3] The abbreviations used are: DR, death receptor; DcR, decoy receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; IAP, inhibitors of apoptosis protein; FasL, Fas ligand; TRAIL-R, TRAIL receptor; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HMEC, human mammary epithelial cell; HRP, horseradish peroxidase; PBST, PBS and 0.01% Tween 20; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPA, RNase protection assay; Act D, actinomycin D; NF-κB, nuclear factor κB; MEF, mouse embryo fibroblast; DKO, double knockout; FLICE, caspase-8; TRADD, TNFR1-associated death domain; RIP, receptor interacting protein; zVADfmk, N-benzyloxycarbonyl-valine-alanine-aspartate fluoromethyl ketone; XIAP, X chromosome-linked inhibitor of apoptosis; CDDP, cisplatin; CIAP, cellular inhibitor of apoptosis; TNF, tumor necrosis factor.

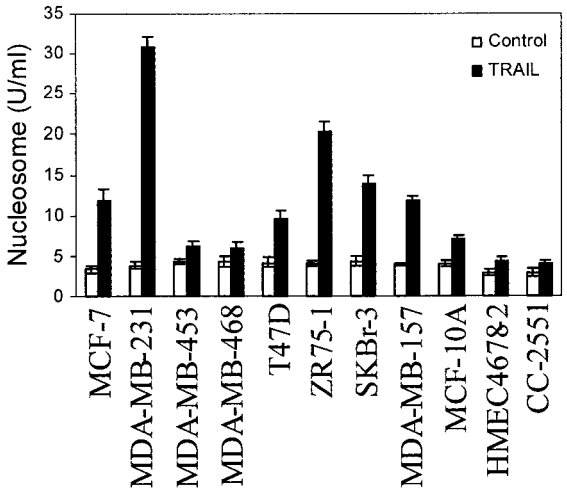

Fig. 1. Effects of TRAIL on apoptosis in human breast normal and malignant cell lines. Breast cell lines were treated with 50 nM TRAIL for 24 h. At the end of incubation, apoptosis was assessed by nucleosome ELISA assay as per the manufacturer's directions. Data represent the mean ± SD from three independent experiments.

MATERIALS AND METHODS

Reagents. Fluorescein-conjugated antibody against caspase-3 was purchased from Cell Signaling (Beverly, MA). Caspase-3, -8, and -9 assay kits; nucleosome ELISA kit; and antibody against tubulin were from Oncogene Research (Cambridge, MA). Antibodies against caspase-3, caspase-9, and PARP and ELISA kits for DR4 and DR5 were purchased from Biosource International, Inc. (Camarillo, CA). Enhanced chemiluminescence Western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL). LipofectAMINE reagent was from Invitrogen (Carlsbad, CA). TRAIL was synthesized as described previously (3). Cell death detection kit (TUNEL) was purchased from Roche Applied Sciences (Indianapolis, IN). RPA kit was purchased from PharMingen (San Diego, CA). All other chemicals used were of analytical grade from Fisher Scientific (Suwanee, GA) or Sigma (St. Louis, MO).

Cells and Culture Conditions. MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-157, ZR75-1, T47D, SKBr3, MCF-10A and MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. HMECs were from Clonetics and grown in culture according to the instructions provided with them. Transfection with Bcl-2 and Bcl-X_L genes has been described previously (19, 20).

RPA. Total RNAs were extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD). The RPA was performed as per the manufacturer's instructions (PharMingen). Briefly, probe set including the DNA templates was used for T7 RNA polymerase-directed synthesis of [^{32}P]UTP-labeled antisense RNA probes. Two μg of RNA were incubated with [α-^{32}P]UTP-labeled single-stranded RNA probes overnight at 56°C and treated with RNase for 45 min at 30°C. The RNA-RNA complexes were resolved by electrophoresis in 6% denaturing polyacrylamide gels and analyzed by autoradiography.

Western Blot Analysis. Cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM sodium orthovanadate, and a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, and 2 μg/ml aprotinin). Lysates were sonicated for 10 s, centrifuged for 20 min at 10,000 × g, and stored at −70°C. Equal amounts of lysate protein were run on 10% SDS-PAGE gels and transferred electrophoretically to nitrocellulose. Nitrocellulose blots were blocked with 6% nonfat dry milk in TBS buffer [20 mM Tris-HCl (pH 7.4) and 500 mM NaCl] and incubated with primary antibody in TBS containing 1% BSA overnight at 4°C. Immunoblots were washed three times (15, 5, and 5 min each) with TBS and 0.01% Tween 20. Immunoreactivity was detected by sequential incubation with HRP-conjugated secondary antibody and enhanced chemiluminescence reagents.

Nucleosome ELISA. In this assay, mono- and oligonucleosomes are captured on precoated DNA-binding proteins. Cells (2×10^6) were seeded into 24-well plates in the presence or absence of various drugs for 36 h (see the figure legends). Cells were harvested for nucleosome ELISA assay according to the manufacturer's directions (Oncogene Research Products). Briefly, anti-histone 3 biotin-labeled antibody binds to the histone component of captured nucleosomes and is detected after incubation with streptavidin-linked HRP conjugate. HRP catalyzes the conversion of colorless tetramethylbenzidine to blue, and addition of a stop solution changes the color to yellow, the intensity of which is proportional to the number of nucleosomes in the sample.

DR4 and DR5 ELISA. Cells were treated with TRAIL for 24 or 48 h. At the end of the incubation period, cells were harvested and washed twice with ice-cold PBS. Cells were lysed in extraction buffer for 30 min on ice with vortexing at 10-min intervals. The extracts were centrifuged at 13,000 rpm for 10 min at 4°C. Lysates were aliquoted and assayed for DR4 and DR5 proteins by ELISA as per the manufacturer's directions (Biosource International, Inc.).

Antitumor Activity of Chemotherapeutic Drugs and TRAIL. Breast cancer MDA-MB-231 cells (5×10^6) were injected in the hind leg of BALB/c *nu/nu* mice (6 weeks old). After tumor formation (100 mm^3), mice (7 mice/group) received injection with vehicle (80% propylene glycol-20% PBS), paclitaxel (15 mg/kg), etoposide (15 mg/kg), camptothecin (15 mg/kg), Adriamycin (15 mg/kg), TRAIL (10 mg/kg), TRAIL after 24 h of paclitaxel administration, TRAIL after 24 h of etoposide administration, TRAIL after 24 h of camptothecin administration, or TRAIL after 24 h of Adriamycin administration. In combination treatments, drugs were administered 24 h before TRAIL treatment because our goal was to induce DRs DR4 and DR5, so that successive treatment with TRAIL would result in enhanced apoptosis of tumor cells. The administration of drugs was carried out once a week for 3 weeks after tumor formation. Drugs were administered s.c. at the site of tumor. Tumor growth was followed by measurements of tumor diameters with a sliding caliper two times a week, and mice survival was monitored daily. The tumor volume (*TV*) was calculated according to the following formula: $TV = L \times W^2/2$, where L and W are the major and minor dimensions, respectively.

Caspase-3 Activity *in Situ*. Tumor tissues derived from mice were fixed with 10% formalin, embedded in paraffin, and sectioned. Slides were washed with PBST three times for 5 min each and blocked with 3% BSA in PBST for

Table 1 *Effects of chemotherapeutic drugs and TRAIL on apoptosis in breast normal and cancer cells*

Breast normal and cancer cells were treated with paclitaxel (0.1 μM), vincristine (0.1 μM), vinblatine (0.1 μM), etoposide (0.1 μM), adriamycin (0.1 μM) and camptothecin (0.1 μM) with or without TRAIL (0.1 μM) for 24 h. Chemotherapeutic drugs and TRAIL were added together. Apoptosis was measured by annexin V-FITC and PI staining.

	CC-2551	MCF-10A	MCF-7	MDA-MB-231	MDA-MB-453	MDA-MB-468	T47D	ZR75-1
Control	4 ± 0.3	5 ± 0.2	4 ± 0.3	3 ± 0.3	4 ± 0.2	3 ± 0.2	4 ± 0.2	4 ± 0.2
Paclitaxel (0.1 μM)	4 ± 0.2	7 ± 0.3	9 ± 0.2	15 ± 0.6	6 ± 0.2	9 ± 0.4	6 ± 0.3	8 ± 0.3
Vincristine (0.1 μM)	6 ± 0.2	6 ± 0.2	8 ± 0.2	14 ± 0.5	9 ± 0.6	8 ± 0.5	13 ± 0.6	12 ± 0.7
Vinblastine (0.1 μM)	6 ± 0.3	7 ± 0.5	8 ± 0.3	14 ± 0.5	8 ± 0.3	9 ± 0.6	11 ± 0.5	11 ± 0.7
Etoposide (0.1 μM)	7 ± 0.4	6 ± 0.2	9 ± 0.2	12 ± 0.5	7 ± 0.4	8 ± 0.5	11 ± 0.5	12 ± 0.5
Adriamycin (0.1 μM)	6 ± 0.3	7 ± 0.4	13 ± 0.3	13 ± 0.7	9 ± 0.6	8 ± 0.6	13 ± 0.6	11 ± 0.7
Camptothecin (0.1 μM)	7 ± 0.4	7 ± 0.3	10 ± 1.5	12 ± 0.5	10 ± 0.6	9 ± 0.6	11 ± 0.8	10 ± 0.5
TRAIL (0.1 μM)	5 ± 0.5	4 ± 0.2	8 ± 0.5	25 ± 1	5 ± 0.2	5 ± 0.3	8 ± 0.6	22 ± 0.6
Paclitaxel + TRAIL	11 ± 0.6	11 ± 0.5	15 ± 0.7	50 ± 2.5	18 ± 1.5	13 ± 0.6	16 ± 0.6	18 ± 1.5
Vincristine + TRAIL	9 ± 0.6	10 ± 0.6	14 ± 0.5	45 ± 3	18 ± 1.5	13 ± 0.6	15 ± 1.5	17 ± 1.2
Vinblastine + TRAIL	11 ± 1	12 ± 1	17 ± 1	43 ± 2.5	17 ± 1	15 ± 0.6	16 ± 1	18 ± 1.2
Etoposide + TRAIL	10 ± 1.2	13 ± 1	20 ± 1	53 ± 4.2	18 ± 0.6	14 ± 0.7	17 ± 2	17 ± 0.7
Adriamycin + TRAIL	13 ± 0.6	13 ± 1	23 ± 1.2	62 ± 3.2	19 ± 0.6	13 ± 0.6	20 ± 1.5	20 ± 1.7
Camptothecin + TRAIL	12 ± 0.6	12 ± 1.5	21 ± 3	50 ± 2.9	17 ± 1	14 ± 0.9	16 ± 2	15 ± 0.6

Table 2 *Sensitization of TRAIL-resistant breast cancer cell lines with chemotherapeutic drugs*

Cells were treated with paclitaxel (0.1 μM), etoposide (0.1 μM), Adriamycin (0.1 μM), and camptothecin (0.1 μM) for 24 h, followed by treatment with or without TRAIL (0.1 μM) for another 24 h. Alternatively, cells were treated with TRAIL (0.1 μM) for 24 h, followed by treatment with or without paclitaxel (0.1 μM), etoposide (0.1 μM), Adriamycin (0.1 μM), or camptothecin (0.1 μM) for 24 h. At the end of incubation period, apoptosis was measured by annexin V-FITC and PI staining.

	MCF-7	MDA-MB-453	MDA-MB-468	T47D
Control	3 ± 0.1	3 ± 0.1	4 ± 0.2	4 ± 0.2
Paclitaxel (0.1 μM), 24 h	9 ± 0.2	6 ± 0.2	7 ± 0.3	6 ± 0.2
Paclitaxel, 24 h→24 h	29 ± 0.6	26 ± 0.5	24 ± 3.8	25 ± 1
Etoposide (0.1 μM), 24 h	12 ± 0.3	8 ± 0.6	9 ± 0.5	10 ± 0.4
Etoposide, 24 h→24 h	31 ± 2.0	24 ± 1.1	28 ± 2.0	26 ± 1.5
Adriamycin (0.1 μM), 24 h	13 ± 0.2	9 ± 0.3	8 ± 0.6	12 ± 0.4
Adriamycin, 24 h→24 h	31 ± 2.5	25 ± 0.7	25 ± 2.3	27 ± 1
Camptothecin (0.1 μM), 24 h	12 ± .5	9 ± 0.6	8 ± 1.5	11 ± 0.4
Camptothecin, 24 h→24 h	27 ± 0.6	21 ± 1	27 ± 2.3	33 ± 1
TRAIL (0.1 μM), 24 h	10 ± 0.2	7 ± 0.4	5 ± 0.2	10 ± 0.4
TRAIL, 24 h→24 h	16 ± 0.5	10 ± 0.5	8 ± 0.3	29 ± 1.5
Paclitaxel, 24 h→TRAIL, 24 h	81 ± 2.5	72 ± 3.6	69 ± 3.1	85 ± 2.3
Etoposide, 24 h→TRAIL, 24 h	78 ± 2.5	73 ± 1.5	73 ± 2.0	75 ± 3.1
Adriamycin, 24 h→TRAIL, 24 h	89 ± 3.1	73 ± 2	78 ± 3.5	82 ± 3.5
Camptothecin, 24 h→TRAIL, 24 h	81 ± 3	70 ± 2.1	68 ± 3.6	69 ± 1.5
TRAIL, 24 h→Paclitaxel, 24 h	32 ± 2.1	24 ± 2	28 ± 3.5	35 ± 1.2
TRAIL, 24 h→Etoposide, 24 h	33 ± 2.1	25 ± 2.1	28 ± 1.6	31 ± 0.8
TRAIL, 24 h→Adriamycin, 24 h	35 ± 1	27 ± 2.1	33 ± 1.0	35 ± 1.3
TRAIL, 24 h→Camptothecin, 24 h	30 ± 2.1	23 ± 1.2	26 ± 1.4	32 ± 1.5

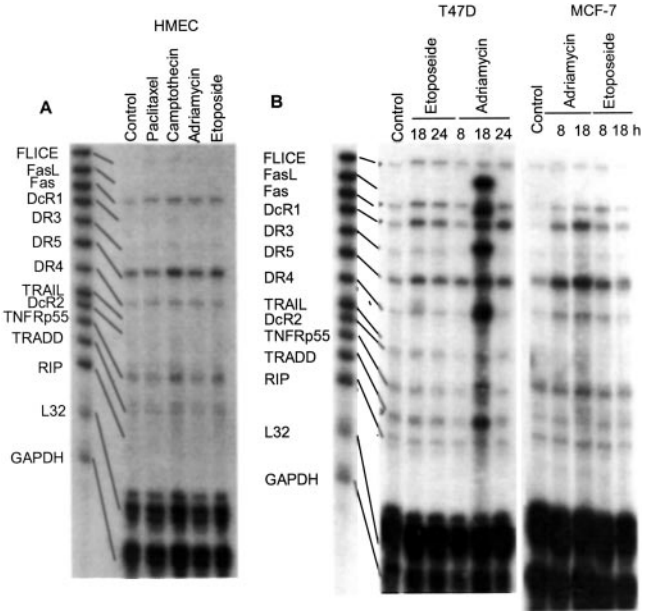

Fig. 2. Effects of chemotherapeutic drugs on the expression of death-related genes in normal HMECs and breast cancer T47D and MCF-7 cells. *A,* HMECs were treated with or without paclitaxel (100 nM), camptothecin (100 nM), Adriamycin (100 nM), and etoposide (100 nM) for 18 h. Total RNA was used in RPA (hAPO-3D; PharMingen) to measure the expression of death-related genes. L32 and GAPDH are shown as housekeeping genes. *B,* T47D and MCF-7 cells were treated with or without etoposide (100 nM) or Adriamycin (100 nM) for various time periods. Total RNA was used in RPA (hAPO-3D; PharMingen) to measure the expression of death-related genes (FLICE, FasL, Fas, DcR1, DR3, DR5, DR4, TRAIL, DcR2, TNFRp55, TRADD and RIP). L32 and GAPDH are shown as housekeeping genes.

1 h at room temperature. Sections were incubated with fluorescent primary antibody in 3% BSA in PBST and PI (0.5 μg/ml) with gentle shaking overnight at 4°C. Sections were washed thrice for 10 min each with PBST and washed once with PBS. Slides were mounted with Gelvatol and visualized under the fluorescence microscope.

TUNEL Assay. Tumor tissues were fixed with 10% formalin, embedded in paraffin, and sectioned. Paraffin-embedded tissue slides were dewaxed and rehydrated by heating at 60°C, followed by washing in xylene and rehydration through a graded series of ethanol and double-distilled water. Tissue sections were incubated for 20 min at 37°C with proteinase K working solution [15 μg/ml in 10 mM Tris-HCl (pH 7.4)] followed by rinsing twice with PBS. After air drying the slides, 50 μl of TUNEL reaction mixture were added on sample and covered with lid, and slides were incubated for 60 min at 37°C in a humidified atmosphere in the dark. Slides were washed thrice with PBS, air dried, mounted, and visualized with a fluorescence microscope.

Flow Cytometric Analysis of Sub-G_1 (Hypodiploid) Apoptotic Cells. Cells were harvested and fixed in 85% ethanol. Cells were then stained with 1 μg/ml PI in PBS with 0.5% NP40 and RNase A. PI-stained cells were analyzed using a Beckton Dickson FACStar flow cytometer. The percentage of sub-G_1 (hypodiploid) apoptotic cells was calculated using ModFit LT.

Assessment of Apoptosis by Annexin V Staining. Cells were resuspended in 100 μl of staining solution (containing annexin V fluorescein and PI in a HEPES buffer; Annexin-V-FLUOS Staining Kit; Boehringer Mannheim). After incubation at room temperature for 20 min, cells were analyzed by flow cytometry. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and PI).

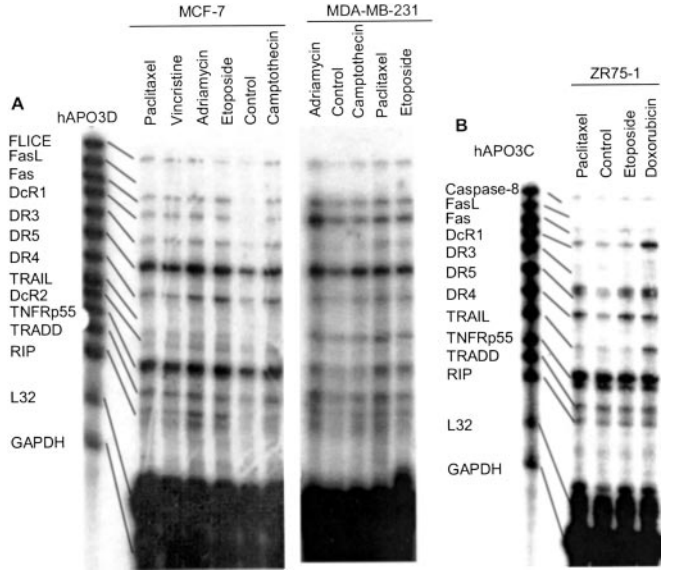

Fig. 3. Effects of chemotherapeutic drugs on the expression of death-related genes in MCF-7, MDA-MB-231, and ZR75-1 cells. *A,* MCF-7 and MDA-MB-231 cells were treated with or without paclitaxel (100 nM), vincristine (100 nM), Adriamycin (100 nM), etoposide (100 nM), and camptothecin (100 nM) for 18 h. Total RNA was used in RPA (hAPO-3D; PharMingen) to measure the expression of death-related genes (FLICE, FasL, Fas, DcR1, DR3, DR5, DR4, TRAIL, DcR2, TNFRp55, TRADD, and RIP). L32 and GAPDH are shown as housekeeping genes. *B,* ZR75-1 cells were treated with or without paclitaxel (100 nM), etoposide (100 nM), and Adriamycin (100 nM) for 18 h. Total RNA was used in RPA (hAPO-3C; PharMingen) to measure the expression of death-related genes (caspase-8, FasL, Fas, DcR1, DR3, DR5, DR4, TRAIL, TNFRp55, TRADD, and RIP). L32 and GAPDH are shown as housekeeping genes.

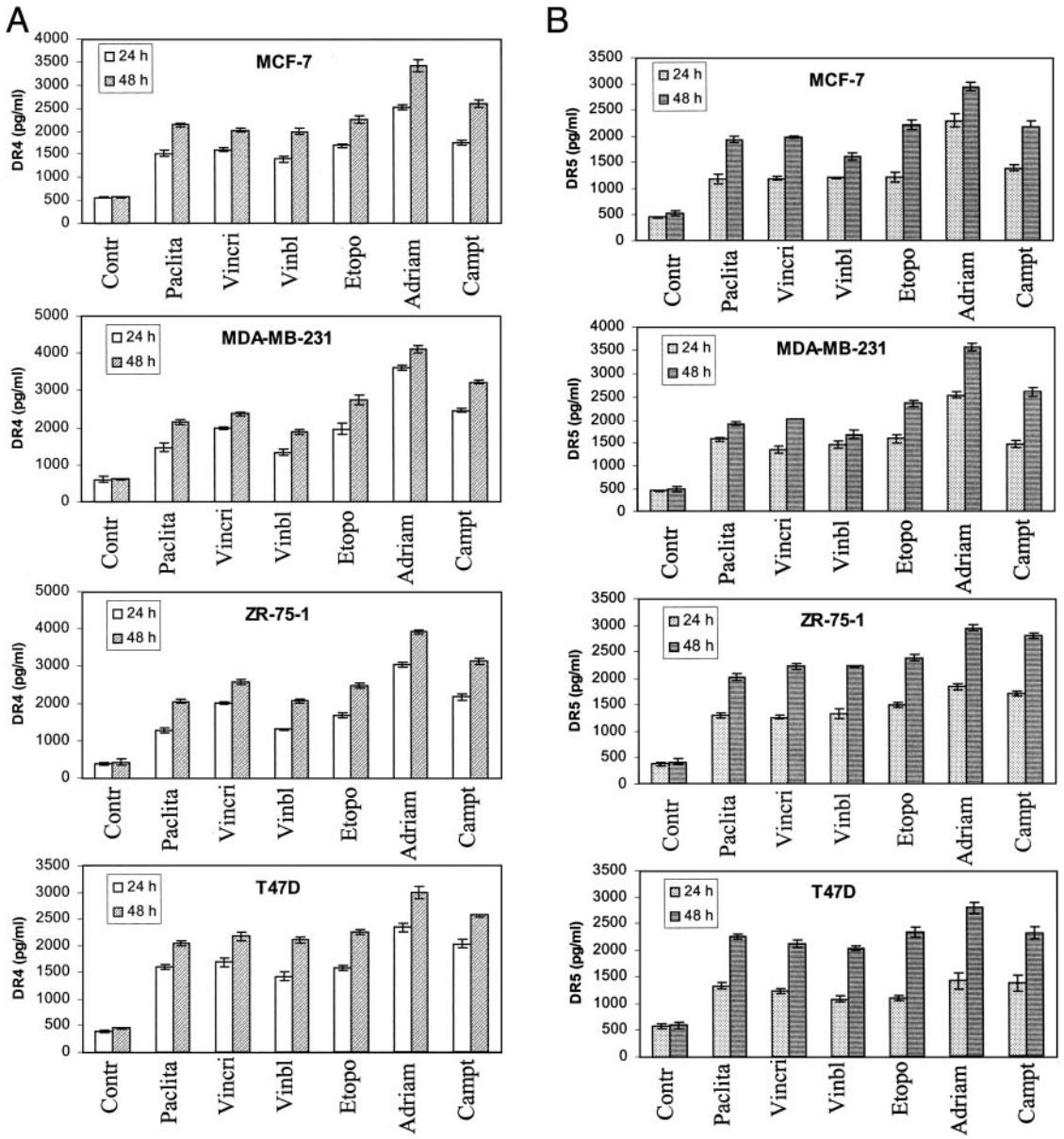

Fig. 4. Effects of chemotherapeutic drugs on the expression of DR4 and DR5 in breast cancer cells. *A,* effects of chemotherapeutic drugs on DR4 protein in MCF-7, MDA-MB-231, ZR75-1, and T47D cells. Breast cancer cells were treated with or without paclitaxel (100 nM), vincristine (100 nM), vinblastine (100 nM), etoposide (100 nM), Adriamycin (100 nM), and camptothecin (100 nM) for 24 or 48 h. Expression of DR4 was measured by ELISA as per the manufacturer's instructions (Biosource International, Inc.). *B,* effects of chemotherapeutic drugs on DR5 protein in MCF-7, MDA-MB-231, ZR75-1, and T47D cells. Breast cancer cells were treated with or without paclitaxel (100 nM), vincristine (100 nM), vinblastine (100 nM), etoposide (100 nM), Adriamycin (100 nM), and camptothecin (100 nM) for 24 or 48 h. Expression of DR5 was measured by ELISA as per the manufacturer's instructions (Biosource International, Inc.).

Statistical Analyses. For each studied variable, mean and SE were calculated. Differences between groups were analyzed by one- or two-way ANOVA. Differences in the rates of complete tumor inhibitions or survivors were validated by χ^2 test. Survival curves were drawn according to Kaplan-Meier analysis.

RESULTS

Effects of TRAIL on Apoptosis in Breast Normal and Malignant Cells. We first examined the effects of TRAIL on primary HMECs (HMEC4678-2 and CC-2551), immortalized nontransformed breast epithelial MCF-10A cells, and breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-157, MDA-MB-453, MDA-MB-468, ZR75-1, T47D, and SKBr3). MDA-MB-231 and ZR75-1 cells were sensitive to TRAIL-induced apoptosis (Fig. 1). By comparison, MCF-7, T47D, SKBr3, MDA-MB-157, and MCF-10A cells were semisensitive, whereas MDA-MB-453, MDA-MB-468, HMEC4678-2, and CC-2551 cells were resistant to TRAIL-induced apoptosis. These data suggest that TRAIL induced apoptosis in normal and malignant breast cells with varying sensitivity.

Effects of Chemotherapeutic Drugs and TRAIL on Apoptosis in Breast Cancer Cells. Chemotherapeutic drugs have been shown to sensitize Fas- and TRAIL-induced apoptosis in breast cancer cell lines (21, 22). Because TRAIL primarily induces apoptosis in cancer cells without appreciable effects in normal cells (1, 4), we sought to examine the interactive effects of anticancer drugs and TRAIL on apoptosis in primary HMECs (CC-2551), immortalized nontransformed breast epithelial cells (MCF-10A), and breast cancer cells (MCF-7, MDA-MB-231, MDA-MB-453, MDA-MB-468, T47D, and ZR75-1). Cells were treated with paclitaxel, vincristine, vinblastine,

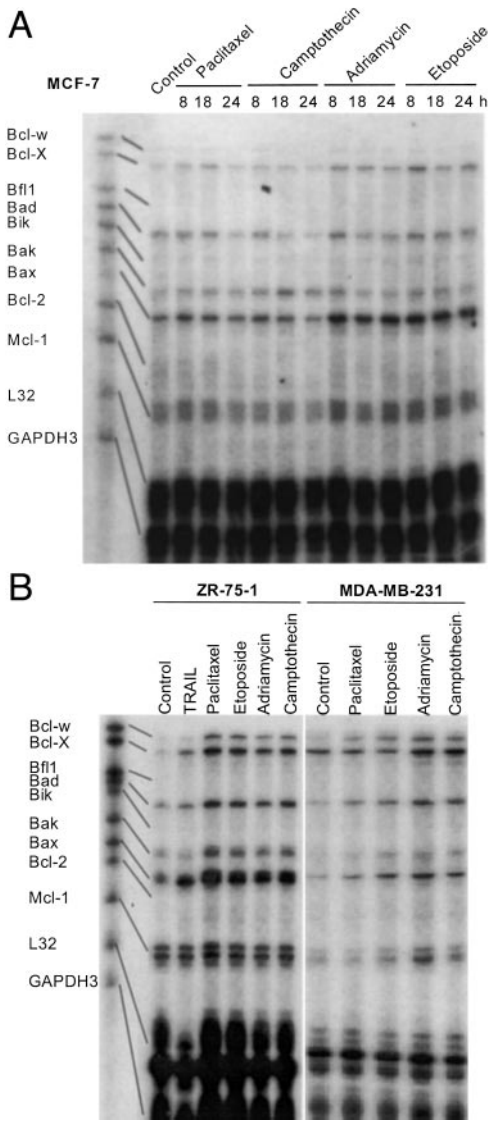

Fig. 5. Effects of chemotherapeutic drugs on the expression of Bcl-2 family members in breast cancer cells. *A,* MCF-7 cells were treated with or without paclitaxel (100 nM), camptothecin (100 nM), Adriamycin (100 nM), and etoposide (100 nM) for 18 h. Total RNA was used in RPA (hAPO-2C; PharMingen) to measure the expression of Bcl-2 family members (Bcl-w, Bcl-x, Bfl1, Bad, Bik, Bak, Bax, Bcl-2, and Mcl-1). L32 and GAPDH are shown as housekeeping genes. *B,* ZR75-1 and MDA-MB-231 cells were treated with or without TRAIL (50 nM), paclitaxel (100 nM), etoposide (100 nM), Adriamycin (100 nM), and camptothecin (100 nM) for 18 h. Total RNA was used in RPA (hAPO-2C; PharMingen) to measure the expression of Bcl-2 family members. L32 and GAPDH are shown as housekeeping genes.

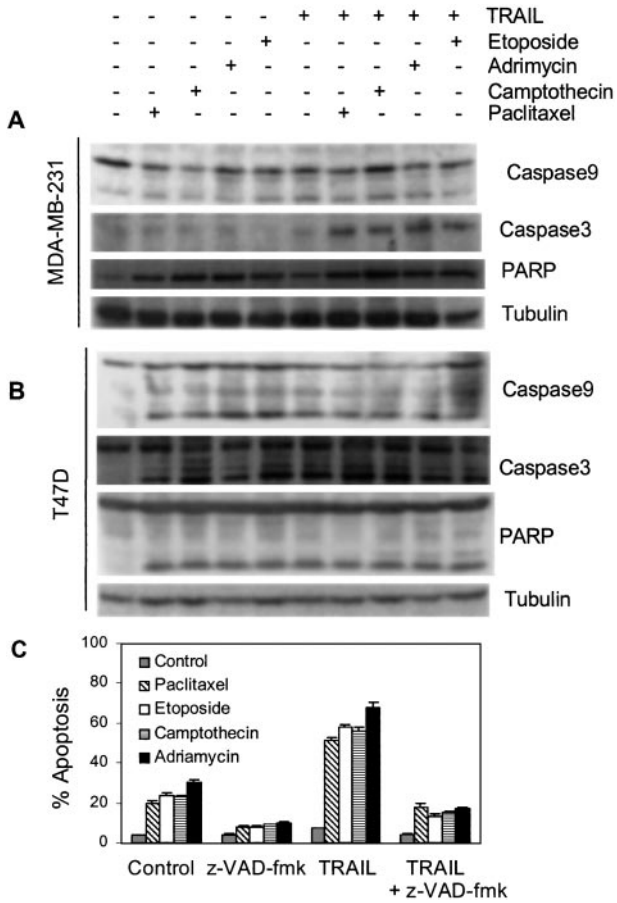

Fig. 7. Involvement of caspase in chemotherapeutic drug- and/or TRAIL-induced apoptosis. *A,* MDA-MB-231 cells were treated with or without paclitaxel (100 nM), camptothecin (100 nM), Adriamycin (100 nM), etoposide (100 nM), and TRAIL (50 nM) for 36 h. Western blot analyses were performed to measure the expression of caspase-9 and caspase-3 and PARP cleavage. Antitubulin antibody was used as a loading control. *B,* T47D cells were treated with or without paclitaxel (100 nM), camptothecin (100 nM), Adriamycin (100 nM), etoposide (100 nM), and TRAIL (50 nM) for 36 h. Western blot analyses were performed to measure the expression of caspase-9 and caspase-3 and PARP cleavage. Antitubulin antibody was used as a loading control. *C,* cells were pretreated with 50 μM ZVAD-fmk for 2 h followed by treatment with paclitaxel (100 nM), etoposide (100 nM), camptothecin (100 nM), or Adriamycin (100 nM) in the presence and absence of TRAIL (50 nM) for 36 h. At the end of incubation period, apoptosis was measured. Data represent the mean ± SE.

etoposide, Adriamycin, and camptothecin in the presence or absence of TRAIL for 24 h, and apoptosis was measured (Table 1). Paclitaxel, vincristine, vinblastine, etoposide, Adriamycin, and camptothecin alone slightly induced apoptosis in all cancer cell lines. Apoptotic

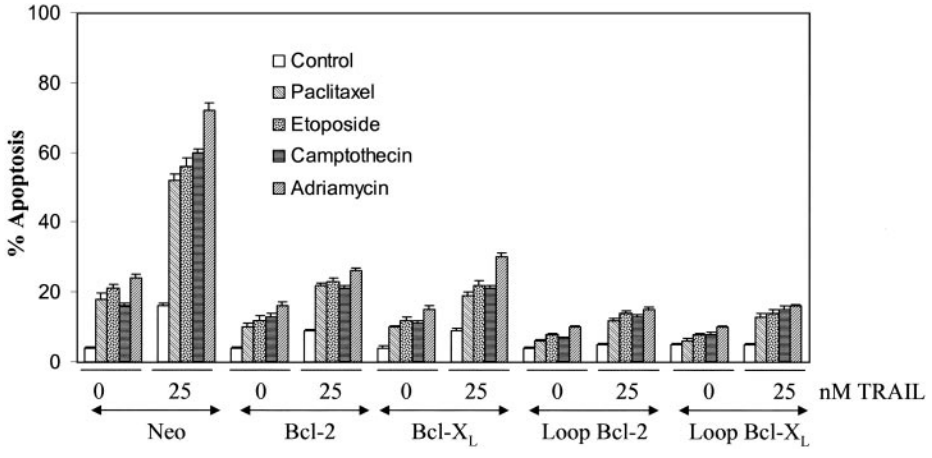

Fig. 6. Effects of Bcl-2, Bcl-X_L, Δloop Bcl-2, and Δloop Bcl-X_L on drug-induced apoptosis. MDA-MB-231 cells were transfected with pSSFV-Neo, pSSFV-Bcl-2, pSSFV-Bcl-X_L, pSSFV/ΔloopBcl-2, and pSSFV/ΔloopBcl-X_L. Transfectants were treated with paclitaxel (100 nM), etoposide (100 nM), camptothecin (100 nM), and Adriamycin (100 nM) with or without TRAIL (50 nM) for 36 h. At the end of incubation period, apoptosis was measured. The data represent the mean ± SE.

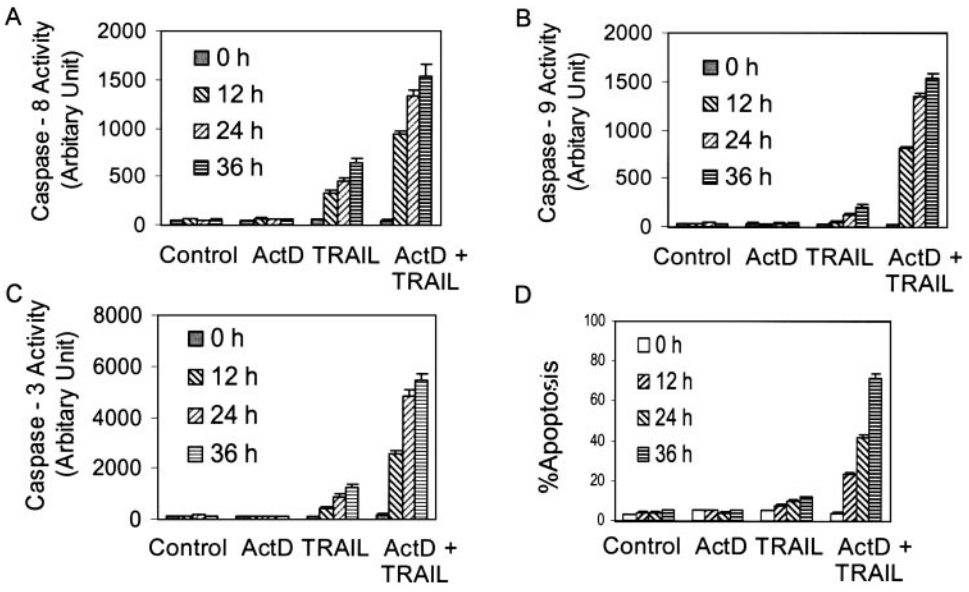

Fig. 8. Combination of Act D and TRAIL induces activation of caspase-8, -9, and -3 and apoptosis. T47D cells were treated with either ActD (100 nM) or TRAIL (50 nM) for 0, 12, 24, or 36 h. In combination treatments, cells were pretreated with Act D (100 nM) for 6 h, followed by TRAIL (50 nM) for 0, 12, 24, or 36 h. At the end of the incubation period, caspase-8, -9, and -3 activity (*A*–*C*) and apoptosis (*D*) were measured. Data represent the mean ± SE.

effects of TRAIL varied among cancer and normal cells; sensitive cell lines were MDA-MB-231 and ZR75-1, semisensitive cell lines were MCF-7 and T47D, and resistant cell lines were CC-2551, MCF-10A, MDA-MB-453, and MDA-MB-468. The concurrent treatment of all cell types with chemotherapeutic drugs plus TRAIL for 24 h also induced significantly more apoptosis than exposure to single drug alone (Table 1) or the sequential exposure to TRAIL followed by drugs (data not shown).

Because most of the breast cancer cell lines tested were either semisensitive or resistant to TRAIL, we next sought to examine whether these cells can be killed by pretreatment with chemotherapeutic drugs followed by TRAIL. Chemotherapeutic drugs, when added several hours before the addition of TRAIL, may increase the apoptotic effects of TRAIL by up-regulating DRs. MCF-7, MDA-MB-453, MDA-MB-468, and T47D cells were pretreated with paclitaxel, vincristine, vinblastine, etoposide, Adriamycin, and camptothecin for 24 h, followed by TRAIL treatment for another 24 h. Interestingly, the pretreatment with chemotherapeutic drugs followed by TRAIL was effective in inducing apoptosis in TRAIL-resistant cells (Table 2). To understand this synergistic interaction, the reverse sequence of drug exposure was used, where cells were pretreated with TRAIL for 24 h, followed by treatment with chemotherapeutic drugs for an additional 24 h. Reverse sequence of drug exposure (TRAIL followed by drug) has resulted in significantly less apoptosis than the sequential treatment of cells with chemotherapeutic drugs followed by TRAIL. These data suggest that sequential treatment with chemotherapeutic drugs followed by TRAIL can be used to kill TRAIL-resistant cells and therefore can be used in combination therapy of breast cancer.

Regulation of DRs by Chemotherapeutic Drugs. TRAIL induces apoptosis by binding to DRs and activating caspase-8 (11, 23, 24). We first examined the effects of chemotherapeutic drugs on the regulation of DR family members in normal HMECs by RPA (hAPO-3D probe set, FLICE, Fas, FasL, DcR1, DR3, DR5, DR4, TRAIL, DcR2, TNFRp55, TRADD, RIP, L32, and GAPDH; PharMingen). Palcitaxel, camptothecin, Adriamycin, and etoposide had no effect on DR4 expression; however, they slightly induced DR5 in normal HMECs (Fig. 2*A*).

Because chemotherapeutic drugs synergize with TRAIL in inducing apoptosis, we sought to examine whether this interaction is due to increased expression of DR4 and/or DR5. The expression of DR family members was analyzed at various time points by RPA (hAPO-3C probe set). Cells were treated with etoposide or Adriamycin for 8, 18, or 24 h (Fig. 2*B*). The maximum induction of DR4 and DR5 by etoposide or Adriamycin was observed at 18 h in T47D and MCF-7 cell lines. Similarly, these chemotherapeutic drugs significantly induced DR5 and slightly induced DR4 expression in MCF-7 and MDA-MB-231 cells (Fig. 3*A*). Paclitaxel, etoposide, and Adriamycin also induced DR4 and DR5 at 18 h in ZR75-1 cells (Fig. 3*B*).

We confirmed the induction of DRs by chemotherapeutic drugs by ELISA (Fig. 4, *A* and *B*). Cells were treated with paclitaxel, vincristine, vinblastine, etoposide, Adriamycin, and camptothecin for 24 and 48 h to measure DR4 and DR5 proteins. These drugs significantly induced DR4 and DR5 in MCF-7, MDA-MB-231, ZR75-1, and T47D cells. The induction of DRs by these drugs appears to be independent of p53 status because p53 wild-type (MCF-7 and ZR75-1) and p53-mutant cell lines (MDA-MB-231 and T47D) were similarly sensitized to TRAIL by chemotherapy.

Effects of Chemotherapeutic Drugs on the Expression of Bcl-2 Family Members. Several reports indicated that chemotherapeutic drugs induce apoptosis by enhancing the expression of proapoptotic members of Bcl-2 family (25–27). We therefore examined the effects of chemotherapeutic drugs on the expression of Bcl-2 family members by RPA (hAPO 2C probe-set; PharMingen; Fig. 5). Paclitaxel, camptothecin, Adriamycin, and etoposide significantly induced Bax expression in MCF-7 cells; Bad, Bax, and Bad expression in ZR75-1 cells; and Bad and Bax expression in MDA-MB-231 cells (Fig. 5, *A* and *B*). These drugs also induced Bcl-X, which was later confirmed to be Bcl-Xs by Western blot analysis (data not shown). These data suggest that proapoptotic proteins may play a role, at least in part, in the synergistic interaction among chemotherapeutic drugs and TRAIL.

Overexpression of Bcl-2 and Bcl-X_L Blocks Synergistic Interactions between Chemotherapeutic Drugs and TRAIL. We and others have shown that Bcl-2 and Bcl-X_L block drug-induced mitochondrial membrane potential and apoptosis by acting at the level of outer mitochondrial membrane (26, 28–31). To assess the involvement of mitochondrial pathway on the synergistic interaction of chemotherapeutic drugs and TRAIL on apoptosis, we used MDA-MB-231 cells overexpressing Bcl-2, Δloop Bcl-2, Bcl-X_L, and Δloop Bcl-X_L (19, 20). Overexpression of Bcl-2, Δloop Bcl-2, Bcl-X_L, or Δloop Bcl-X_L inhibited paclitaxel, etoposide,

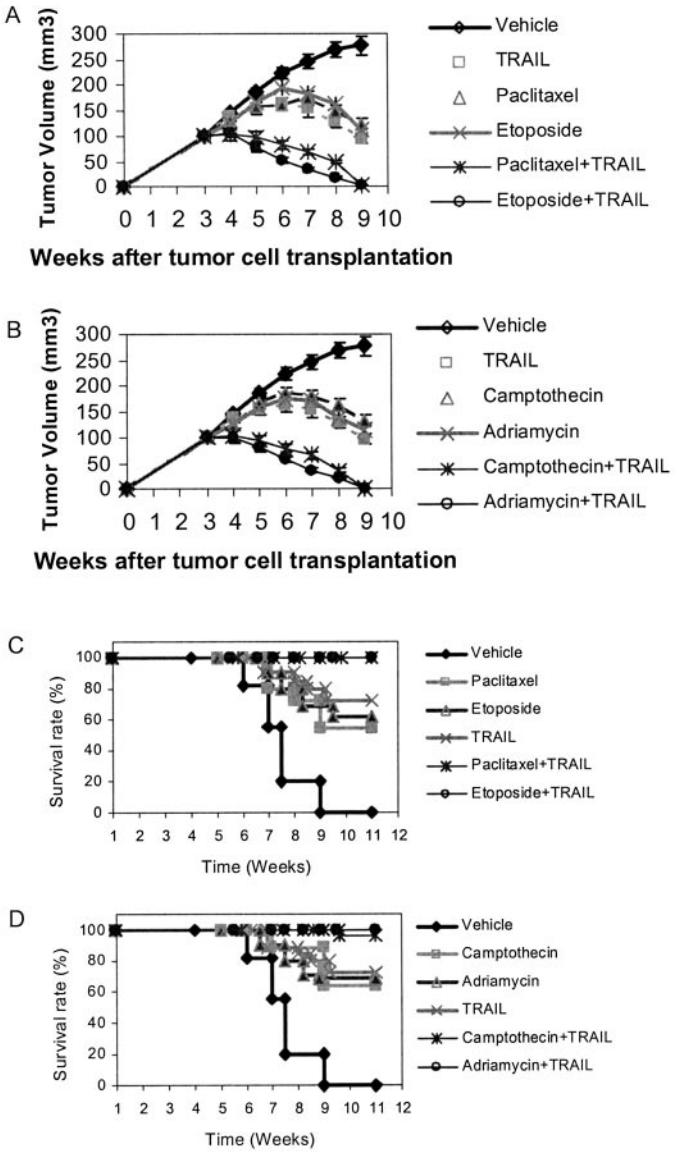

Fig. 9. Synergistic effects of chemotherapeutic drugs with TRAIL on tumor growth and survival of tumor-bearing mice. *A,* interactive effects of paclitaxel and etoposide with TRAIL on tumor volumes at different times after tumor cell transplantation in mice. Breast cancer MDA-MB-231 cells (5×10^6) were injected in the hind leg of Balb/c nude mice. After tumor formation (100 mm^3), mice received injection with vehicle, paclitaxel, etoposide, TRAIL, paclitaxel 24 h followed by TRAIL, or etoposide 24 h followed by TRAIL. Drugs were administered s.c. at the site of tumor. Tumor volume was measured weekly. Data represent the mean ± SE. *B,* interactive effects of camptothecin and Adriamycin with TRAIL on tumor volumes. After tumor formation, tumor-bearing mice received injection with vehicle, camptothecin, Adriamycin, TRAIL, camptothecin for 24 h followed by TRAIL, or Adriamycin for 24 h followed by TRAIL. Data represent the mean ± SE. *C,* Kaplan-Meier analysis of survival after treatment of mice with paclitaxel and etoposide with TRAIL. Independent groups of tumor-bearing mice were treated as described in *A*. Survival was monitored daily. *D,* Kaplan-Meier analysis of survival after treatment of mice with camptothecin and Adriamycin with TRAIL. Independent groups of tumor-bearing mice were treated as described in *B*. Survival was monitored daily.

camptothecin, Adriamycin, and TRAIL-induced apoptosis (Fig. 6). Overexpression of Bcl-2, Δloop Bcl-2, Bcl-X_L, or Δloop Bcl-X_L also inhibited apoptosis when cells were pretreated with paclitaxel, etoposide, camptothecin, and Adriamycin followed by treatment with TRAIL. These data suggest that the mitochondrial pathway plays a major role in apoptosis induced by the synergistic interactions of chemotherapeutic drugs and TRAIL because Bcl-2 and Bcl-X_L proteins mainly exert their biological effects by anchoring on the mitochondrial membrane.

Chemotherapeutic Drugs Augment TRAIL-induced Apoptosis through Caspase Activation. Caspase activation appears to be a common pathway in apoptosis induced by stress stimuli in many systems (32–34). Because chemotherapeutic drugs augment TRAIL-induced apoptosis, we sought to examine the mechanism of this interaction by measuring caspase-9 and -3 activation by their cleavage. Activated caspase cleaves substrate PARP, which can be used to confirm apoptosis (35, 36). MDA-MB-231 and T47D cells were treated with etoposide, Adriamycin, camptothecin, and paclitaxel with or without TRAIL for 36 h, and cleavage of caspase-9, caspase-3, and PARP was determined by Western blot analysis (Fig. 7). Treatment of cells with chemotherapeutic drugs with or without TRAIL resulted in caspase-9, caspase-3, and PARP cleavage (Fig. 7, *A* and *B*). To identify whether the augmentation of TRAIL-induced apoptosis by chemotherapy was mediated through caspase activation, the TRAIL-resistant T47D cell line was incubated with chemotherapeutic drugs, TRAIL, or the combination of chemotherapeutic drugs plus TRAIL in the presence or absence of the caspase inhibitor zVAD-fmk (Fig. 7*C*). The inhibition of caspase activity by zVAD-fmk significantly blocked apoptosis induced by chemotherapeutic drugs alone, TRAIL alone, and the combination of drugs and TRAIL. These data suggest that caspase activation plays a significant role in the synergistic interaction among chemotherapeutic drugs and TRAIL.

Pretreatment with Act D, Followed by Treatment with TRAIL, Activates Caspase-8, -9, and -3 and Induces Apoptosis. We and others have shown that RNA and protein synthesis inhibitors sensitize TRAIL-resistant prostate and lung cancer cells to undergo apoptosis (5, 37, 38). We therefore examined whether pretreatment with Act D followed by TRAIL results in apoptosis of TRAIL-resistant cells through activation of caspase-8, -9, and -3. T47D cells were pretreated with Act D (100 nM) for 6 h, followed by treatment with TRAIL (100 nM) for various time periods (0, 12, 24, and 36 h) to measure the activities of caspase-8, -9, and -3 and apoptosis (Fig. 8). Act D was ineffective in inducing caspase activity and apoptosis. TRAIL slightly induced caspase -8, -9, and -3 activity (although cells were resistant to TRAIL; Fig. 8, *A*–*D*). Pretreatment of T47D cells with Act D (100 nM) for 6 h followed by treatment with TRAIL activated caspase-3, -9, and -8 and induced apoptosis. Treatment of cells with Act D resulted in down-regulation of XIAP and up-regulation of Bcl-Xs proteins (data not shown). These data suggest that activation of caspase-8 is required but not sufficient to induce apoptosis in TRAIL-resistant cells, and pretreatment of these cells with Act D sensitizes cells to undergo apoptosis upon TRAIL treatment.

Effects of Chemotherapeutic Drugs and TRAIL on Tumor Growth and Survival of Tumor-bearing Athymic Nude Mice. Because chemotherapeutic drugs enhanced the apoptosis-inducing potential of TRAIL by up-regulating DRs DR4 and DR5, we sought to examine whether this combination is effective in a breast cancer xenograft model *in vivo*. MDA-MB-231 cells were implanted into the right thigh of athymic nude mice. Three weeks after transplantation, mice received injection with vehicle (80% propylene glycol-20% PBS), paclitaxel, etoposide, camptothecin, Adriamycin, and paclitaxel followed by TRAIL; etoposide followed by TRAIL; camptothecin followed by TRAIL; and Adriamycin followed by TRAIL three times during 3 weeks. In combination treatment, TRAIL was injected 24 h after each drug administration because, based on *in vitro* data, synergy was achieved when TRAIL was added 24 h after drug treatment. Administration of paclitaxel, etoposide, camptothecin, or Adriamycin resulted in inhibition of tumor growth and enhancement of survival of tumor-bearing mice (Fig. 9). Interestingly, sequential treatment with chemotherapeutic drugs followed by TRAIL administration synergistically inhibited tumor growth and enhanced survival of mice. No

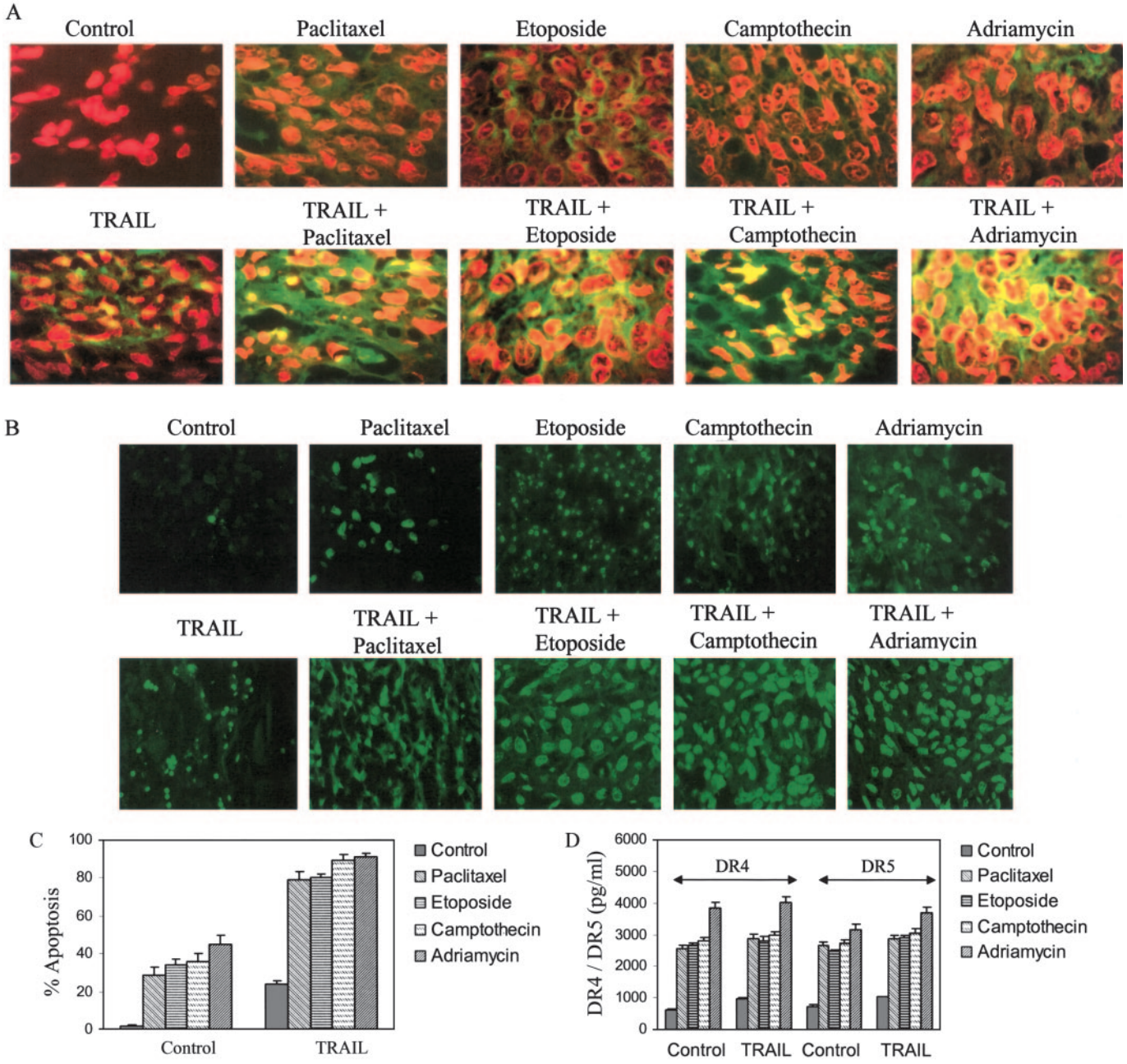

Fig. 10. Effects of chemotherapeutic drugs and TRAIL on caspase-3 activation, apoptosis, and expression of DRs in tumor tissues. Mice were pretreated with drugs followed by TRAIL administration as described in the Fig. 9 legend. *A,* caspase-3 activity in formalin-fixed tumor tissues derived from mice. *B,* TUNEL staining of formalin-fixed tumor tissues. TUNEL staining was performed as per the manufacturer's instructions (PharMingen). *C,* interactive effects of chemotherapeutic drugs and TRAIL on apoptosis as measured by TUNEL staining. *D,* expression of DRs DR4 and DR5 in tumor tissues.

toxicity was observed in the liver and brain tissues of mice. These data suggest that the sequential administration of chemotherapeutic drugs followed by TRAIL may be a viable option to treat breast cancer patients.

Effects of Chemotherapeutic Drugs and TRAIL on Caspase-3 Activity, Apoptosis, and DR4/DR5 Expression in Tumor Tissues Derived from Nude Mice. Because the combination of chemotherapeutic drugs and TRAIL was effective in regressing tumor growth and enhancing survival of mice, we sought to examine caspase-3 activity and apoptosis in formalin-fixed tumor tissues (Fig. 10). Tumor tissues derived from mice treated with paclitaxel, etoposide, camptothecin, Adriamycin, and TRAIL alone showed enhanced caspase-3 activity and apoptosis compared with control (Fig. 10, *A*−*C*). Sequential treatment of mice with chemotherapeutic drugs followed by TRAIL revealed significantly higher caspase-3 activity and apoptosis compared with single-agent treatment alone. Increased caspase-3 activity and tumor cell apoptosis correlated with reduction in tumor volume, as shown in Fig. 9. Paclitaxel, etoposide, camptothecin, and Adriamycin enhanced DR4 and DR5 expression in tumor cells, and the combination of these drugs with TRAIL had no further effect on DRs (Fig. 10*D*). These data suggest that the synergistic interaction between these drugs and TRAIL on apoptosis is due to induction, at least in part, of DRs by drugs. These data suggest that sequential treatment with chemotherapeutic drugs followed by TRAIL administration may be a viable option to treat breast cancer patients.

DISCUSSION

The present results indicate that TRAIL induces apoptosis in breast cancer cells with varying sensitivity. Because death signals originate from the DRs, their up-regulation in cancer cells can enhance ligand-induced cytotoxicity. Here we show that chemotherapeutic drugs (paclitaxel, vincristine, vinblastine, etoposide, Adriamycin, and camptothecin) induce expression of DR4 and/or DR5, and successive treatment with TRAIL results in apoptosis in an additive or synergistic manner *in vitro*. Similar to our studies, the combination of TRAIL and Act D induces apoptosis in TRAIL-resistant human pancreatic cancer cells (39). Previous studies have demonstrated that chemotherapeutic drugs and TRAIL induce apoptosis in several cancer cells *in vitro* (6–8, 21, 22, 40, 41), but their combined effects (chemotherapy followed by TRAIL) have not been tested in a xenograft model system. In our study, the combination treatment is effective in a human xenograft model system, in which treatment with chemotherapeutic drugs followed by TRAIL results in regression of tumor growth and enhancement of survival of tumor-bearing nude mice. Similarly, treatment of mice with systemic administration of TRAIL plus CDDP caused significant suppression of both tumor formation and growth of established human U87MG xenografts in a synergistic fashion (42). Our studies suggest that the sequential administration of chemotherapeutic drugs followed by TRAIL can be used in combination therapy of breast cancer patients.

Up-regulation of DR4 and DR5 enhanced the responsiveness of cells to TRAIL (4). Most importantly, pretreatment of cells with chemotherapeutic drugs followed by TRAIL causes a synergistic apoptotic response. Among all of the chemotherapeutic drugs tested *in vitro* and *in vivo*, Adriamycin is the most effective in up-regulating DR4 and DR5 and inducing apoptosis when combined with TRAIL. Similarly, chemotherapeutic drugs up-regulate DR4 and/or DR5 expression, thereby enhancing TRAIL-induced apoptosis *in vitro* (6, 8, 21, 43, 44). Furthermore, TRAIL induces apoptosis in Adriamycin-resistant MCF-7 cells already expressing high levels of DRs DR4 and DR5 (data not shown), suggesting that TRAIL can be used to treat breast cancer patients who have acquired resistance to Adriamycin. Thus, an increase in DR4 and/or DR5 levels by chemotherapeutic drugs is capable of enhancing apoptosis in response to added TRAIL in both TRAIL-sensitive and TRAIL-/Adriamycin-resistant cancer cells.

Breast cancer cell lines are sensitized to TRAIL-induced apoptosis by chemotherapeutic agents (most effectively by Adriamycin) with different sensitivity. Similarly, cancer cells can be sensitized to Fas-induced apoptosis by chemotherapeutic agents in others tissues, in part by up-regulation of Fas (45–47). Our data indicate that sensitization of breast cells to TRAIL-induced apoptosis is independent of p53 mutation state, suggesting the possibility that the p53 signaling pathway is not involved in the up-regulation of DR4 and/or DR5. We have demonstrated that cells harboring wild-type (HMEC, ZR75-1, and MCF-7) and mutant (T47D, MDA-MB-231, and MDA-MB-468) p53 (48–50) can be sensitized by chemotherapeutic drugs. Similarly, in other studies, DR5 is regulated by chemotherapeutic drugs independent of p53 status, consistent with the suggestion that there are p53-independent pathways regulating DR expression (6, 42). These data suggest that the combination treatment approach could target breast cancer cells harboring both wild-type and mutant p53. Regulation of DR expression may also depend on the type of insult because the mode of actions of drugs used are distinct (microtubule- and DNA-damaging drugs), and the level of DR up-regulation varied among them. It is possible that DNA lesions preferentially initiate downstream pathways that activate the transcription machinery for DR expression. The other possible candidate is the transcription factor NF-κB (51, 52). The oncogenic role of NF-κB was observed in leukemia and lymphoma (53), breast cancer (52, 54), and pancreatic cancer (55). We have recently shown that overexpression of RelA subunit of NF-κB inhibits caspase-8 and DR4 and DR5 expression and enhances expression of cIAP1 and cIAP2 after TRAIL treatment (52). By comparison, overexpression of c-Rel enhances DR4, DR5, and Bcl-Xs and inhibits cIAP1, cIAP2, and survivin after TRAIL treatment. We proposed that the RelA subunit acts as a survival factor by inhibiting expression of DR4/DR5 and caspase-8 and up-regulating cIAP1 and cIAP2 (52). Thus, the dual function of NF-κB as an inhibitor or activator of apoptosis depends on the relative levels of RelA and c-Rel subunits.

Altered regulation of apoptosis has been linked to the development of cancer (56), and mitochondria have emerged as gatekeepers in many apoptotic signaling pathways (57–59). Members of the Bcl-2 family of proteins that regulate apoptotic signaling through mitochondria are key regulators of apoptosis in mammalian development, and their deregulation is associated with disease, particularly cancer (27). There are three classes of Bcl-2 family members: (*a*) apoptosis promoters (*e.g.,* Bax and Bak); (*b*) apoptosis inhibitors (*e.g.,* Bcl-2, Bcl-X_L, and adenoviral E1B 19K); and (*c*) the BH3-only Bcl-2 family members (*e.g.,* Bid, Puma, Noxa, Bad, and Nbk/Bik), which contain the BH3 interaction domain, that act as apoptosis promoters and inhibitors (27). Signal transduction events modify the activity of BH3-only proteins, which in turn interact with pro- or antiapoptotic family members to either antagonize or activate their function. Stimulation of apoptosis can therefore be achieved by activating a death activity or by antagonizing a survival activity.

In our studies, chemotherapeutic drugs induced proapoptotic molecules Bax and Bak. Wild-type MEFs, transformed MEFs (58, 60, 61), or transformed baby mouse kidney epithelial cell lines (62) derived from $Bax^{-/-}$ and/or $Bak^{-/-}$ deficient mice have been useful in determining their role in apoptosis. Similar to wild-type cells, $Bax^{-/-}$ or $Bak^{-/-}$ MEFs still release mitochondrial protein cytochrome *c* and undergo apoptosis in response to cytotoxic agents and DR signaling, whereas $Bax^{-/-}$ and $Bak^{-/-}$ DKO MEFs are profoundly defective (58, 59, 62). Thus, Bax or Bak functions in a redundant capacity to facilitate the release of cytochrome *c* from the intermembrane space (58, 59, 62). We have recently shown that, unlike cytochrome *c* release, TRAIL-induced Smac/DIABLO release was blocked in $Bax^{-/-}$, $Bak^{-/-}$, or $Bax^{-/-}$ and $Bak^{-/-}$ DKO MEFs, suggesting the differential regulation of these mitochondrial proteins during apoptosis (59). $Bax^{-/-}$ and $Bak^{-/-}$ DKO MEFs are also resistant to death signaling by overexpression of BH3-only proteins, indicating that they are required downstream components of these signaling pathways (60, 61). Inactivation of both Bax and Bak was required for tumor growth and was selected for *in vivo* during tumorigenesis (62).

We have shown previously (24) that TRAIL-induced apoptosis requires mitochondria. The activation of caspase-8 by TRAIL is necessary but may not be sufficient to induce apoptosis. Cross-talk between the DR and mitochondrial pathways is mediated by caspase-8 cleavage of Bid (24, 63–65). tBid activates proapoptotic members Bax and Bak to release cytochrome *c* from mitochondria (58). Bcl-2 and Bcl-X_L inhibit chemotherapy and/or TRAIL-induced apoptosis by blocking cytochrome *c* release (19, 60). In epithelial cells, mitochondria appear to amplify the apoptotic signals leading to activation of caspase-9 (66). Caspase-9, in turn, activates downstream caspases and the cleavage of apoptotic substrates that mediate the dismantling of the cell (66). The synergistic effects of chemotherapeutic drugs and TRAIL on apoptosis occur through activation of downstream caspase-3, which can be activated by both mitochondria-dependent and -independent pathways (23). Because MCF-7 cells lack caspase-3 expression due to a genetic deletion of its gene (67), in this cell line

other downstream caspases such as caspase-6 or caspase-7 may replace the role of caspase-3.

In summary, we have developed a strategy of combining chemotherapeutic drugs with TRAIL for the treatment of human breast cancer. We have shown *in vitro* and *in vivo* that chemotherapeutic drugs up-regulate DR4 and/or DR5 and that subsequent treatment with TRAIL induces apoptosis in cell lines and tumor cells and enhances survival of tumor-bearing mice. These studies provide a foundation for the development of combined treatment regimens that would enhance the apoptotic response to TRAIL in cancer patients.

REFERENCES

1. Ashkenazi, A., Pai, R. C., Fong, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Koumenis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrokh, Z., and Schwall, R. H. Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Investig., *104:* 155–162, 1999.
2. Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat. Med., *5:* 157–163, 1999.
3. Kim, E. J., Suliman, A., Lam, A., and Srivastava, R. K. Failure of Bcl-2 to block mitochondrial dysfunction during TRAIL-induced apoptosis. Tumor necrosis-related apoptosis-inducing ligand. Int. J. Oncol., *18:* 187–194, 2001.
4. Srivastava, R. K. TRAIL/Apo-2L: mechanisms and clinical applications in cancer. Neoplasia, *3:* 535–546, 2001.
5. Kandasamy, K., and Srivastava, R. K. Role of the phosphatidylinositol 3′-kinase/PTEN/Akt kinase pathway in tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in non-small cell lung cancer cells. Cancer Res., *62:* 4929–4937, 2002.
6. Keane, M. M., Ettenberg, S. A., Nau, M. M., Russell, E. K., and Lipkowitz, S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res., *59:* 734–741, 1999.
7. Nimmanapalli, R., Perkins, C. L., Orlando, M., O'Bryan, E., Nguyen, D., and Bhalla, K. N. Pretreatment with paclitaxel enhances apo-2 ligand/tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis of prostate cancer cells by inducing death receptors 4 and 5 protein levels. Cancer Res., *61:* 759–763, 2001.
8. Munshi, A., McDonnell, T. J., and Meyn, R. E. Chemotherapeutic agents enhance TRAIL-induced apoptosis in prostate cancer cells. Cancer Chemother. Pharmacol., *50:* 46–52, 2002.
9. Gura, T. How TRAIL kills cancer cells, but not normal cells. Science (Wash. DC), *277:* 768, 1997.
10. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science (Wash. DC), *277:* 818–821, 1997.
11. Pan, G., O'Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, R., Ni, J., and Dixit, V. M. The receptor for the cytotoxic ligand TRAIL. Science (Wash. DC), *276:* 111–113, 1997.
12. French, L. E., and Tschopp, J. The TRAIL to selective tumor death. Nat. Med., *5:* 146–147, 1999.
13. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. Science (Wash. DC), *277:* 815–818, 1997.
14. Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Boiani, N., Timour, M. S., Gerhart, M. J., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J., *16:* 5386–5397, 1997.
15. Degli-Esposti, M. A., Smolak, P. J., Walczak, H., Waugh, J., Huang, C. P., DuBose, R. F., Goodwin, R. G., and Smith, C. A. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J. Exp. Med., *186:* 1165–1170, 1997.
16. Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., and Goodwin, R. G. The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity, *7:* 813–820, 1997.
17. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Huang, A., Skubatch, M., Baldwin, D., Yuan, J., Gurney, A., Goddard, A. D., Godowski, P., and Ashkenazi, A. A novel receptor for Apo2L/TRAIL contains a truncated death domain. Curr. Biol., *7:* 1003–1006, 1997.
18. Emery, J. G., McDonnell, P., Burke, M. B., Deen, K. C., Lyn, S., Silverman, C., Dul, E., Appelbaum, E. R., Eichman, C., DiPrinzio, R., Dodds, R. A., James, I. E., Rosenberg, M., Lee, J. C., and Young, P. R. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J. Biol. Chem., *273:* 14363–14367, 1998.
19. Srivastava, R. K., Sollott, S. J., Khan, L., Hansford, R., Lakatta, E. G., and Longo, D. L. Bcl-2 and Bcl-X_L block thapsigargin-induced nitric oxide generation, c-Jun NH_2-terminal kinase activity, and apoptosis. Mol. Cell. Biol., *19:* 5659–5674, 1999.
20. Srivastava, R. K., Mi, Q. S., Hardwick, J. M., and Longo, D. L. Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. Proc. Natl. Acad. Sci. USA, *96:* 3775–3780, 1999.
21. Gibson, S. B., Oyer, R., Spalding, A. C., Anderson, S. M., and Johnson, G. L. Increased expression of death receptors 4 and 5 synergizes the apoptosis response to combined treatment with etoposide and TRAIL. Mol. Cell. Biol., *20:* 205–212, 2000.
22. Mizutani, Y., Nakao, M., Ogawa, O., Yoshida, O., Bonavida, B., and Miki, T. Enhanced sensitivity of bladder cancer cells to tumor necrosis factor related apoptosis inducing ligand mediated apoptosis by cisplatin and carboplatin. J. Urol., *165:* 263–270, 2001.
23. Srivastava, R. K. Intracellular mechanisms of TRAIL and its role in cancer therapy. Mol. Cell. Biol. Res. Commun., *4:* 67–75, 2000.
24. Suliman, A., Lam, A., Datta, R., and Srivastava, R. K. Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways. Oncogene, *20:* 2122–2133, 2001.
25. Green, D. R., Bissonnette, R. P., and Cotter, T. G. Apoptosis and cancer. Important Adv. Oncol., *8:* 37–52, 1994.
26. Srivastava, R. K., Srivastava, A. R., Korsmeyer, S. J., Nesterova, M., Cho-Chung, Y. S., and Longo, D. L. Involvement of microtubules in the regulation of Bcl2 phosphorylation and apoptosis through cyclic AMP-dependent protein kinase. Mol. Cell. Biol., *18:* 3509–3517, 1998.
27. Gross, A., McDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13:* 1899–1911, 1999.
28. Miyashita, T., and Reed, J. C. Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. Blood, *81:* 151–157, 1993.
29. Ibrado, A. M., Huang, Y., Fang, G., Liu, L., and Bhalla, K. Overexpression of Bcl-2 or Bcl-xL inhibits Ara-C-induced CPP32/Yama protease activity and apoptosis of human acute myelogenous leukemia HL- 60 cells. Cancer Res., *56:* 4743–4748, 1996.
30. Reed, J. C. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. Hematol. Oncol. Clin. N. Am., *9:* 451–473, 1995.
31. Srivastava, R. K., Sasaki, C. Y., Hardwick, J. M., and Longo, D. L. Bcl-2-mediated drug resistance: inhibition of apoptosis by blocking nuclear factor of activated T lymphocytes (NFAT)-induced Fas ligand transcription. J. Exp. Med., *190:* 253–265, 1999.
32. Thornberry, N. A. The caspase family of cysteine proteases. Br. Med. Bull., *53:* 478–490, 1997.
33. Nicholson, D. W., and Thornberry, N. A. Caspases: killer proteases. Trends Biochem. Sci., *22:* 299–306, 1997.
34. Salvesen, G. S., and Dixit, V. M. Caspases: intracellular signaling by proteolysis. Cell, *91:* 443–446, 1997.
35. Kaufmann, S. H., Desnoyers, S., Ottaviano, Y., Davidson, N. E., and Poirier, G. G. Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res., *53:* 3976–3985, 1993.
36. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature (Lond.), *371:* 346–347, 1994.
37. Chen, X., Thakkar, H., Tyan, F., Gim, S., Robinson, H., Lee, C., Pandey, S. K., Nwokorie, C., Onwudiwe, N., and Srivastava, R. K. Constitutively active Akt is an important regulator of TRAIL sensitivity in prostate cancer. Oncogene, *20:* 6073–6083, 2001.
38. Nesterov, A., Lu, X., Johnson, M., Miller, G. J., Ivashchenko, Y., and Kraft, A. S. Elevated AKT activity protects the prostate cancer cell line LNCaP from TRAIL-induced apoptosis. J. Biol. Chem., *276:* 10767–10774, 2001.
39. Matsuzaki, H., Schmied, B. M., Ulrich, A., Standop, J., Schneider, M. B., Batra, S. K., Picha, K. S., and Pour, P. M. Combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and actinomycin D induces apoptosis even in TRAIL-resistant human pancreatic cancer cells. Clin. Cancer Res., *7:* 407–414, 2001.
40. Nagane, M., Huang, H. J., and Cavenee, W. K. The potential of TRAIL for cancer chemotherapy. Apoptosis, *6:* 191–197, 2001.
41. Lacour, S., Hammann, A., Wotawa, A., Corcos, L., Solary, E., and Dimanche-Boitrel, M. T. Anticancer agents sensitize tumor cells to tumor necrosis factor- related apoptosis-inducing ligand-mediated caspase-8 activation and apoptosis. Cancer Res., *61:* 1645–1651, 2001.
42. Nagane, M., Pan, G., Weddle, J. J., Dixit, V. M., Cavenee, W. K., and Huang, H. J. Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand *in vitro* and *in vivo*. Cancer Res., *60:* 847–853, 2000.
43. Nimmanapalli, R., Porosnicu, M., Nguyen, D., Worthington, E., O'Bryan, E., Perkins, C., and Bhalla, K. Cotreatment with STI-571 enhances tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL or apo-2L)-induced apoptosis of Bcr-Abl-positive human acute leukemia cells. Clin. Cancer Res., *7:* 350–357, 2001.
44. Wen, J., Ramadevi, N., Nguyen, D., Perkins, C., Worthington, E., and Bhalla, K. Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. Blood, *96:* 3900–3906, 2000.
45. Friesen, C., Herr, I., Krammer, P. H., and Debatin, K. M. Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. Nat. Med., *2:* 574–577, 1996.
46. Muller, M., Strand, S., Hug, H., Heinemann, E. M., Walczak, H., Hofmann, W. J., Stremmel, W., Krammer, P. H., and Galle, P. R. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO- 1/Fas) receptor/ligand system and involves activation of wild-type p53. J. Clin. Investig., *99:* 403–413, 1997.
47. Fulda, S., Scaffidi, C., Pietsch, T., Krammer, P. H., Peter, M. E., and Debatin, K. M. Activation of the CD95 (APO-1/Fas) pathway in drug- and γ-irradiation-induced apoptosis of brain tumor cells. Cell Death Differ., *5:* 884–893, 1998.
48. Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene, *5:* 893–899, 1990.

49. Vojtesek, B., and Lane, D. P. Regulation of p53 protein expression in human breast cancer cell lines. J. Cell Sci., *105:* 607–612, 1993.
50. Runnebaum, I. B., Yee, J. K., Kieback, D. G., Sukumar, S., and Friedmann, T. Wild-type p53 suppresses the malignant phenotype in breast cancer cells containing mutant p53 alleles. Anticancer Res., *14:* 1137–1144, 1994.
51. Ravi, R., Bedi, G. C., Engstrom, L. W., Zeng, Q., Mookerjee, B., Gelinas, C., Fuchs, E. J., and Bedi, A. Regulation of death receptor expression and TRAIL/Apo2L-induced apoptosis by NF-κB. Nat. Cell. Biol., *3:* 409–416, 2001.
52. Chen, X., Kandasamy, K., and Srivastava, R. K. Differential roles of RelA (p65) and c-Rel subunits of nuclear factor κB in tumor necrosis factor-related apoptosis-inducing ligand signaling. Cancer Res., *63:* 1059–1066, 2003.
53. Gil, J., Rullas, J., Garcia, M. A., Alcami, J., and Esteban, M. The catalytic activity of dsRNA-dependent protein kinase, PKR, is required for NF-κB activation. Oncogene, *20:* 385–394, 2001.
54. Sovak, M. A., Bellas, R. E., Kim, D. W., Zanieski, G. J., Rogers, A. E., Traish, A. M., and Sonenshein, G. E. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Investig., *100:* 2952–2960, 1997.
55. Wang, W., Abbruzzese, J. L., Evans, D. B., Larry, L., Cleary, K. R., and Chiao, P. J. The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin. Cancer Res., *5:* 119–127, 1999.
56. Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. Apoptosis: a link between cancer genetics and chemotherapy. Cell, *108:* 153–164, 2002.
57. Wang, X. The expanding role of mitochondria in apoptosis. Genes Dev., *15:* 2922–2933, 2001.
58. Wei, M. C., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Korsmeyer, S. J. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science (Wash. DC), *292:* 727–730, 2001.
59. Kandasamy, K., Srinivasula, S. M., Alnemri, E. S., Thompson, C. B., Korsmeyer, S. J., Bryant, J. L., and Srivastava, R. K. Involvement of proapoptotic molecules Bax and Bak in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced mitochondrial disruption and apoptosis: differential regulation of cytochrome *c* and Smac/DIABLO release. Cancer Res., *63:* 1712–1721, 2003.
60. Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. BCL-2. BCL-X_L sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol. Cell, *8:* 705–711, 2001.
61. Zong, W. X., Lindsten, T., Ross, A. J., MacGregor, G. R., and Thompson, C. B. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev., *15:* 1481–1486, 2001.
62. Degenhardt, K., Sundararajan, R., Lindsten, T., Thompson, C., and White, E. Bax and Bak independently promote cytochrome *c* release from mitochondria. J. Biol. Chem., *277:* 14127–14134, 2002.
63. Li, H., Zhu, H., Xu, C. J., and Yuan, J. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell, *94:* 491–501, 1998.
64. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl2 interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell, *94:* 481–490, 1998.
65. Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. Caspase cleaved BID targets mitochondria and is required for cytochrome *c* release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J. Biol. Chem., *274:* 1156–1163, 1999.
66. Cryns, V., and Yuan, J. Proteases to die for. Genes Dev., *12:* 1551–1570, 1998.
67. Kurokawa, H., Nishio, K., Fukumoto, H., Tomonari, A., Suzuki, T., and Saijo, N. Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells. Oncol. Rep., *6:* 33–37, 1999.