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

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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 DRs 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-2; 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/Dec1/LIT; Refs. 10, 13, and 15), TRAIL-R4 (TRUND/Dec2; Refs. 16 and 17), and osteoproterein (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 DecRs, whereas the fifth receptor, osteoproterein, 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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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 embryonic fibroblast; DKO, double knockout; FLICE, caspase-8; TRADD, TNFR1-Associated Death Domain; RIP, receptor interacting protein; NAVδfnsk, N-β-hexyloxy-carbonyl-valine-alanine-aspartate fluromethyl 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 ng/ml 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 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 × 10⁴) were injected in the hind leg of BALB/c nude mice (6 weeks old). After tumor formation (100 mm³), 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 × W²/2, 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 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 nm Tris-HCl (pH 7.6), 150 nm 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 × 10⁶) 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.

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

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

Flow Cytometric Analysis of Sub-G1 (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-G1 (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).

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.
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 χ² 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,
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. 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, Bcl-X, Bak, Bax, Bcl-2, and Mcl-1). L32 and GAPDH are shown as housekeeping genes.

Fig. 6. Effects of Bcl-2, Bcl-XL, Δloop Bcl-2, and Δloop Bcl-XL on drug-induced apoptosis. MDA-MB-231 cells were transfected with pSSFV-Neo, pSSFV-Bcl-2, pSSFV-Bcl-XL, pSSFV/ΔloopBcl-2, and pSSFV/ΔloopBcl-XL. 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. 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.
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 (TRAIL followed by drug) has resulted in significantly less apoptosis than exposure to single drug alone (Table 1) or the sequential exposure to TRAIL followed by drugs (data not shown).

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 chemotherapeutic drugs.

**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-3C probe set). Cells were treated with etoposide or Adriamycin for 8, 18, or 24 h (Fig. 2B). 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. 3A). Paclitaxel, etoposide, and Adriamycin also induced DR4 and DR5 at 18 h in ZR75-1 cells (Fig. 3B).

Overexpression of Bcl-2 and Bcl-X<sub>L</sub> Blocks Synergistic Interactions between Chemotherapeutic Drugs and TRAIL. We and others have shown that Bcl-2 and Bcl-X<sub>L</sub> 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<sub>L</sub>, and Δloop Bcl-X<sub>L</sub> (19, 20). Overexpression of Bcl-2, Δloop Bcl-2, Δloop Bcl-X<sub>L</sub>, and Δloop Bcl-X<sub>L</sub> inhibited paclitaxel, etoposide,
After tumor formation (100 mm³), mice received injection with vehicle, paclitaxel, etoposide, camptothecin, Adriamycin, and TRAIL. Independent groups of tumor-bearing mice were treated as described in the Methods section. Treatment of mice with camptothecin and Adriamycin with TRAIL resulted in inhibition of tumor growth and enhancement of survival of mice. No significant difference was observed in tumor growth or survival when TRAIL was administered alone or as a combination with chemotherapeutic drugs. These data suggest that activation 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.

**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 treatment with TRAIL. Pretreatment with Act D, followed by treatment with TRAIL, activates caspase-8, -9, and -3 and induces apoptosis. 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.

Chemotherapeutic Drugs Enhance 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. 7C). 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 resulted 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 with Act D sensitizes cells to undergo apoptosis upon TRAIL treatment.

**Chemotherapeutic Drugs Augment TRAIL-Induced Apoptosis and TRAIL-Induced Apoptosis**

Camptothecin, Adriamycin, and TRAIL-induced apoptosis (Fig. 6). Overexpression of Bcl-2, Δloop Bcl-2, Bcl-XL, or Δloop Bcl-XL 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-XL proteins mainly exert their biological effects by anchoring on the mitochondrial membrane.
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. 10D). 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 sensitivities. 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 status, 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-XL, and adenosiral 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-deficient 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, 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 regimes that would enhance the apoptotic response to TRAIL in cancer patients.

REFERENCES


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


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