Chemotherapy Augments TRAIL-induced Apoptosis in Breast Cell Lines

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ABSTRACT

Expression and function of the TRAIL apoptotic pathway was investigated in normal and malignant breast epithelial cells. Glutathione-S-transferase (GST)-TRAIL extracellular domain fusion proteins were produced to analyze TRAIL-induced apoptosis. Only GST-TRAIL constructs containing regions homologous to the Fas self-association and ligand binding domains could induce apoptosis. GST-TRAIL induced significant (>90%) apoptosis in just one of eight normal and one of eight malignant breast cell lines. All other lines were relatively resistant to TRAIL-induced apoptosis. Activating TRAIL receptors DR4 and DR5 were expressed in all normal and malignant breast cell lines. The inhibitory receptor TRID was highly expressed in one of four normal and two of seven malignant breast cell lines. DR4, DR5, or TRID expression did not correlate with sensitivity to TRAIL-induced apoptosis. Incubation of cell lines with doxorubicin or 5-fluorouracil significantly augmented TRAIL-induced apoptosis in most breast cell lines. By fractional inhibition analysis, the toxicity of the combination of TRAIL and doxorubicin or 5-fluorouracil was synergistic compared with either agent alone. In contrast, melphalan and paclitaxel augmented TRAIL-induced apoptosis in few cell lines, and methotrexate did not augment it in any cell line. Augmentation of TRAIL-induced apoptosis by doxorubicin or 5-fluorouracil was mediated through caspase activation. This was evidenced by the fact that chemotherapy agents that synergized with TRAIL (e.g., doxorubicin) themselves caused cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), and their toxicity was blocked by the caspase inhibitor Z-Val-Ala-Asp(OMe)-CH$_2$ (ZVAD-fmk). The combination of TRAIL and doxorubicin or 5-fluorouracil was synergistic compared with the inhibitory receptor TRID. In contrast, chemotherapy agents that did not augment TRAIL-induced apoptosis (e.g., methotrexate) caused minimal caspase-3 and PARP cleavage and toxicity. These drugs also did not increase caspase-3 or PARP cleavage when combined with TRAIL. In summary, few breast cell lines are sensitive to TRAIL-induced apoptosis, and no difference in sensitivity is found between normal and malignant cell lines. Treatment with chemotherapy provides an approach to sensitize breast cancer cells to TRAIL-induced apoptosis.

INTRODUCTION

Dysregulation of normal apoptotic mechanisms provides a growth advantage to cancer cells (1–3). In breast cancer, dysregulated apoptotic pathways include down-regulated death receptor pathway function (4), p53 mutations (5–7), and abnormal bcl2 pathway function (8–11). Furthermore, breast cancer treatments including chemotherapy, radiation therapy and hormone therapy induce apoptotic mechanisms to cause cancer cell death (3, 11, 12). Therefore, activation of specific apoptotic mechanisms in breast cancer cells could be an effective means to treat breast cancer.

Because of their expression on cell membranes, activation of death receptors in the TNF$^2$ receptor superfamily provides a specific mechanism to induce apoptosis in breast cancer cells (13). For example, the Fas receptor is a TNF family protein that is expressed on breast cancer cell membranes (4). The Fas pathway can also be activated to induce apoptosis of breast cancer cells in vitro (4). However, the therapeutic usefulness of the Fas pathway is hampered by Fas expression on hepatocytes that causes lethal hepatic apoptosis when the pathway is activated (14). DR4 (also called TRAIL-R1; Ref. 15) and DR5 (also called TRAIL-R2/TRICK-2/KILLER; Refs. 16–22) are other members of the TNF receptor family which are activated by binding the ligand TRAIL (also called Apo-2 L; Refs. 23 and 24). Activation of this pathway causes apoptosis mediated through caspase activation (15–17, 23, 25). Regulation of this pathway is in part controlled through the relative levels of the death receptors DR4 and DR5 and the inhibitory receptor TRID (also called DcR1/TRAIL-R3/LIT; Refs. 16, 17, 20, 22, and 26). Specifically, expression of TRID in cell lines has been reported to correlate with resistance to TRAIL-mediated apoptosis (16, 17). Moreover, TRID is highly expressed in normal tissues, whereas it has substantially lower expression in malignant cell lines. In contrast, expression of DR4 and DR5 is similar in normal tissues and in malignant cell lines (16, 17). Thus, TRAIL may activate apoptosis selectively in breast cancer cells while sparing normal cells (27).

Here the TRAIL pathway is evaluated in normal and malignant breast epithelial cells. The expression and function of the TRAIL pathway in breast cell lines are analyzed. In addition, mechanisms to activate the TRAIL pathway in resistant breast cells are investigated.

MATERIALS AND METHODS

Cell Lines. The MCF10A, 184B5, ZR75-1, T47D, MDA-MB-231, MDA-MB-453, MDA-MB-468, MDA-MB-157, and SKBr-3 cell lines were obtained from American Type Culture Collection (Rockville, MD) and grown in culture according to the instructions provided with them. The MCF7 cell line was a gift provided by Dr. Ed Chu and cultured in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. HMECs were obtained from Cloletics (San Diego, CA) and grown in culture according to the instructions provided with them.

TRAIL and Chemotherapy-mediated Toxicity. To assess TRAIL-mediated cytotoxicity, cells were plated at 5 × 10$^4$ cells/well in 96-well microtiter plates and allowed to adhere to the plate overnight. Freshly eluted GST-TRAIL fusion proteins at the indicated concentrations were added, and the cells were then incubated for an additional 16 h. Cell viability was assessed by MTT dye reduction assay as described previously (4). GST-TRAIL-treated cells were compared with cells incubated with GST protein at the same concentration.

To assess the effects of chemotherapy on TRAIL-mediated apoptosis, cells were plated and allowed to adhere overnight. TRAIL (25 μM) and chemotherapy agents at the indicated concentrations were added to the wells and incubated for an additional 16 h. Cell viability was then assessed by MTT assay as before. Chemotherapy agents used were doxorubicin (Pharmacia & Upjohn, Kalamazoo, MI), 5-fluorouracil (Hoffman-La Roche, Inc., Nutley, NJ), paclitaxel (Bristol-Myers Squibb Co., Princeton, NJ), and melphalan (Glaxo

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Wellcome, Inc., Research Triangle Park, NC), and methotrexate (Immunex Corp., Seattle, WA).

All MTT experiments were performed in quadruplicate and repeated at least three times. Results of representative experiments are given as the mean ± SD and of multiple experiments as the mean ± SE.

Experiments to evaluate RNA and protein and to perform TUNEL assays were carried out in 100-mm tissue culture dishes with 5 × 10^5 cells/dish and the same concentrations of TRAIL and chemotherapy as in the microtiter plates.

The tetrapeptide caspase inhibitor ZVAD-fmk (Enzyme Systems Products., Livermore, CA) was resuspended in DMSO (Sigma Chemical Co., St. Louis, MO) and added to cells at a final concentration of 50 μM 30 min before TRAIL or chemotherapy agents. Control cells were incubated with DMSO at the same concentration as test cells. Cell viability was analyzed by MTT assay after 16 h of incubation with test reagents.

TUNEL assay to detect fragmented DNA in situ was performed on cell cytospins using the In situ Cell Death Detection kit (Boehringer Mannheim, Indianapolis, IN). To demonstrate nuclear morphology, cells were counterstained by mounting with Vectashield mounting medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA).

Isolation and Analysis of RNA and Protein. RNA was extracted from cell lines using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD), and conditions were as recommended by the supplier. RNase protection analysis was performed using 20 μg of total RNA and the Riboquant assay (Pharmingen, San Diego, CA) according to the manufacturer’s recommendation.

Protein was extracted from cells by detergent lysis (1% Triton-X 100, 10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 2 mM sodium vanadate, 5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 10 μg/μl 4–2-aminoethyl-benzenesulfonyl fluoride). Protein lysates were cleared of debris by centrifugation at 15,000 × g for 10 min at 4°C, and concentration was assessed by Bio-Rad colorometric assay (Bio-Rad, Hercules, CA). Protein samples were boiled in an equal volume of sample buffer (20% glycerol, 4% SDS, 0.2% bromophenol blue, 125 mM Tris-HCl, and 640 mM β-mercaptoethanol), fractionated by 10% SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). PARP protein was detected using a polyclonal rabbit anti-PARP antibody (H-250; Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml. Caspase-3 protein was detected using a rabbit polyclonal anti-caspase-3 antibody at 1 μg/ml (65906E; Pharmingen, San Diego, CA). ERK-2 protein was detected using a rabbit polyclonal anti-ERK-2 antibody (C-14; Santa Cruz Biotechnology) at 1 μg/ml. Goat anti-rabbit antibody conjugated with horseradish peroxidase (Amersham, Arlington Heights, IL) was used to visualize immunoreactive proteins at a 1:5000 dilution using SuperSignal (Pierce, Rockford, IL) detection reagent.

TRAIL-GST Fusion Protein Production. TRAIL cDNAs were generated by RT-PCR. First-strand cDNAs were synthesized from 10 μg of spleen total RNA in 20-μl reactions using an oligo(dT) primer in the presence or absence of Moloney murine leukemia virus Reverse Transcriptase (Life Technologies) under conditions described by the supplier. First-strand TRAIL cDNAs (2 μl) were amplified using AmpliTaq DNA polymerase (Perkin-Elmer Corp., Foster City, CA) in 100-μl reaction volumes under standard conditions recommended by the supplier. The mixtures were denatured for 3 min at 95°C, followed by 30 thermal cycles (30 s at 95°C, 30 s at 50°C, and 2 min at 72°C). The 5’-TRAIL primers were linked with BamHI and designed to clone in-frame with the GST sequence of the pGEX-2TK vector (Pharmacia Biotech, Inc., Piscataway, NJ). 3’ TRAIL primers were linked with EcoRI. 5’ TRAIL primers corresponding to the nucleotide sequences for amino acids 95–102, 119–126, 153–160, and 201–210 and 3’ TRAIL primers corresponding to the nucleotide sequences for amino acids 274–281, 222–229, and 184–191 were derived from the published sequences for human TRAIL (oligonucleotide sequences available on request; Refs. 23 and 24). TRAIL cDNA PCR products were gel purified and ligated into the pGEX-2TK plasmid. Their identity was confirmed by sequencing (T7 Sequenase; version 2.0; Amersham).

TRAIL cDNA plasmids were transformed into DH5α Escherichia coli, and GST-TRAIL fusion protein expression was induced with 100 μM isopropylthio-β-D-galactosidase (Pharmacia Biotech, Inc.). Bacteria were harvested and lysed by sonication in 0.1% TONE buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.1% n-octyl β-D-glucopyranoside). GST-TRAIL proteins were purified by precipitation with glutathione-Sepharose beads and then eluted from the beads with 20 mM glutathione (pH 8.5). TONE 0.1% buffer was exchanged for PBS using a PD10 Sephadex column (Pharmacia Biotech, Inc.). Fusion proteins were analyzed by fractionation on 10% SDS-PAGE and visualized with Chromaphor reagent (Promega Corp., Madison, WI). Fusion protein concentrations were measured using a Bio-Rad colorometric assay (Bio-Rad, Hercules, CA). GST-TRAIL proteins were stable when stored bound to glutathione-Sepharose beads (Pharmacia Biotech, Inc.) at 4°C for up to 3 months. All experiments were performed with freshly eluted GST-TRAIL proteins because TRAIL activity was decreased by storage at 4°C to ~75% at 24 h and ~50% at 48 h after elution.

Statistical Analysis. Statistical comparison of mean values was performed using the t test. All P values were two tailed. Interactions between TRAIL and chemotherapy agents were classified by the Fractional Inhibition Method as follows: when expressed as the fractional inhibition of cell viability, additive inhibition produced by both inhibitors (i) occurs when i1 × i2 + i1 + i2; synergism when i1 × i2 > i1 + i2; and antagonism when i1 × i2 < i1 + i2 (28). The concentrations of reagents that induced a 50% reduction in cell viability (IC50) were determined from curves of reagent concentration versus cell viability at 16 h of incubation for each cell line analyzed.

RESULTS

GST-TRAIL Induces Apoptosis in MDA-MB-231 Cells. A GST-TRAIL fusion protein containing amino acids 95–281 of the TRAIL extracellular domain was constructed and used to investigate TRAIL function in breast cell lines. This GST-TRAIL protein induced apoptosis in MDA-MB-231 cells. In time-course experiments, apoptosis was evident in cells incubated with GST-TRAIL by ~4 h and was ~95% complete by 16 h. Therefore, a 16-h overnight incubation time was chosen for additional experiments to analyze TRAIL toxicity in breast cell lines. Apoptosis induced in MDA-MB-231 cells by GST-TRAIL at 16 h is demonstrated by TUNEL assay in Fig. 1A. Morphological changes of apoptosis including nuclear fragmentation and chromatin clumping are also demonstrated in the nuclei of these cells by DAPI staining (Fig. 1A).

To quantitate apoptosis in the MDA-MB-231 cell line, an MTT assay was performed on cells incubated for 16 h with GST-TRAIL. The IC50 for GST-TRAIL was 0.27 μg/ml, and there was <5% cell viability at 5 μg/ml (Fig. 1B). In contrast, GST alone had minimal toxicity in MDA-MB-231 cells.

To investigate if smaller fragments of the TRAIL protein could also induce apoptosis, five additional GST-TRAIL extracellular domain fusion proteins were produced (Fig. 1C). These constructs represent serial deletions of regions in the TRAIL extracellular domain homologous to other TNF ligand family members (e.g., murine TRAIL, human Fas ligand, and TNF ligand; Ref. 23). Only GST-TRAIL fusion constructs amino acids 95–281 and 119–281 were capable of inducing apoptosis in MDA-MB-231 cells. In contrast, GST-TRAIL constructs with greater NH2-terminal deletions (amino acids 153–281 and 203–281) or with any COOH-terminal deletions (amino acids 95–229 and 95–191) were incapable of inducing apoptosis in MDA-MB-231. Subsequently, all experiments were performed with the GST-TRAIL construct amino acids 95–281 (henceforth called TRAIL) at a concentration of 25 μg/ml.

TRAIL Function in Breast Cell Lines. Sixteen breast cell lines including six primary HMECs, two immortalized nontransformed breast epithelial cell lines (184B5 and MCF10A), and eight breast cancer cell lines (ZR75–1, T47D, MCF7, MDA-MB-231, MDA-MB-453, MDA-MB-468, SKBr-3, and MDA-MB-157) were assessed for sensitivity to TRAIL-induced apoptosis (Fig. 2). Only one nontransformed breast cell line (MCF10A) and one breast cancer cell line (MDA-MB-231) were significantly sensitive to TRAIL-induced apoptosis. By contrast, all other lines were relatively resistant to TRAIL-induced apoptosis with 65–100% viability compared with control cells. In the resistant cell lines, even 10-fold higher concentrations of TRAIL failed to induce a greater degree of apoptosis.
TRAIL Receptor Expression in Breast Cell Lines. DR4, DR5, and TRID expression were analyzed in TRAIL-sensitive and -resistant cell lines (Fig. 3). The activating death receptors DR4 and DR5 were expressed in all cell lines analyzed. Compared with other cell lines, expression of DR4 was highest in the TRAIL-sensitive cell line MDA-MB-231 and lowest in the TRAIL-resistant cell line T47D. Expression of DR5 was also highest in the MDA-MB-231 cell line compared with other lines. There was high expression of the inhibitory receptor TRID in the TRAIL-resistant cell lines HMEC 2595-2 and T47D and also in the TRAIL-sensitive cell line MDA-MB-231.
Lower levels of TRID expression were seen in all other cell lines except MDA-MB-453, where expression was absent. Expression of other apoptosis-associated molecules including caspases (caspase-1 through caspase-10a) and bcl2 family members (bclx L/S, bfl1, bik, bak, bax, and bcl2) was also analyzed by RNase protection in these cell lines (data not shown). Expression data did not reveal any consistent difference between the two TRAIL-sensitive breast cell lines (MCF10A and MDA-MB-231) and the nine TRAIL-resistant cell lines. They also did not reveal any difference between normal and malignant breast cell lines.

**TRAIL and Chemotherapy in Breast Cell Lines.** To overcome resistance of most breast cell lines to TRAIL-induced apoptosis, several reagents were tested. IFN-γ in combination with protein synthesis inhibitors (cyclohexamide or actinomycin D) has been demonstrated to overcome resistance to Fas-induced apoptosis in breast cell lines (4). However, this combination had no effect on resistance to TRAIL-induced apoptosis in the same breast cell lines (data not shown).

Chemotherapy has also been shown to sensitize other tissues to Fas-induced apoptosis (29–37). Therefore, breast cell lines were incubated with TRAIL and a variety of chemotherapy agents that are in common clinical use for breast cancer, specifically doxorubicin, 5-fluorouracil, methotrexate, and paclitaxel. Cyclophosphamide, a commonly used alkylating agent, requires hepatic metabolism for activation; therefore, melphalan was substituted as an example of an alkylating agent for this analysis (38).

The toxicity of chemotherapy agents incubated alone or in combination with a fixed concentration TRAIL on the TRAIL-resistant MDA-MB-453 cell line was evaluated (Fig. 4). The data demonstrate that the toxicity of the combination of TRAIL and doxorubicin is significantly greater than the toxicity of each agent alone. With TRAIL alone, only ~25% of MDA-MB-453 cells undergo apoptosis, and an IC_{50} is not reached. The IC_{50} of doxorubicin alone in MDA-MB-453 is ~10 μM. However, the IC_{50} of doxorubicin when combined with TRAIL is ~0.25 μM. A similar augmentation of toxicity is demonstrated for TRAIL combined with 5-fluorouracil. By fractional inhibition analysis, the toxic effect of the combination of TRAIL and doxorubicin or 5-fluorouracil is synergistic compared with each agent alone (28). In contrast, there is no synergy when TRAIL is combined with paclitaxel, melphalan, or methotrexate in MDA-MB-453 cells.

All of the resistant breast cell lines were then analyzed to see whether chemotherapy agents augmented TRAIL-induced apoptosis. The results are summarized in Table 1. Representative data demonstrating results for all resistant breast cell lines incubated with TRAIL, doxorubicin, or methotrexate, and the combination of TRAIL and doxorubicin or methotrexate, are shown in Fig. 5. TRAIL-induced apoptosis was significantly augmented by doxorubicin and by 5-fluorouracil in most breast cell lines (10 of 11 and 8 of 10, respectively; Table 1 and Fig. 5). In contrast, TRAIL-induced apoptosis was not augmented by methotrexate in any cell line. TRAIL-induced apoptosis was augmented by melphalan and paclitaxel in few (4 of 10 and 1 of 10, respectively) breast cell lines.

In general, cell lines sensitized to TRAIL-induced apoptosis by doxorubicin or 5-fluorouracil overlapped; however, there were some specific differences. In particular, SKBr-3 was the only cell line not sensitized to TRAIL-induced apoptosis by doxorubicin, but it was sensitized by 5-fluorouracil. Similarly, the T47D and MCF7 cell lines were not sensitized to TRAIL by 5-fluorouracil, but they were sensitized by doxorubicin. Also of note, both normal breast cell lines and breast cancer cell lines were sensitized to TRAIL-induced apoptosis.
by chemotherapy agents. In fact, melphalan sensitized three of four normal cell lines and only one of six cancer cell lines (Table 1).

Molecular characteristics that correlate with development of sensitivity to TRAIL-induced apoptosis were analyzed in breast cells incubated with and without doxorubicin (5 μM). No consistent change in expression of any TRAIL receptor was identified that could explain the increased sensitivity to TRAIL-induced apoptosis caused by doxorubicin (Fig. 6). Contrary to expected results, expression of the inhibitory receptor TRID was significantly induced by doxorubicin in the ZR75-1 and T47D cell lines and to a lesser extent in the MDA-MB-468 cell line. In addition, no change in expression of caspases (caspase-1 through caspase-10a) or of bcl2 family members (bclx L/S, bfl1, bik, bak, bax, and bcl2) was identified that correlated with development of sensitivity to TRAIL-induced apoptosis (data not shown).

**Chemotherapy Augments TRAIL Toxicity through Caspase Activation.** Caspase activation is the final common pathway to induction of apoptosis in many systems (39). To identify whether the augmentation of TRAIL-induced apoptosis by chemotherapy was mediated through caspase activation, the TRAIL-resistant MDA-MB-453 cell line was incubated with TRAIL, doxorubicin, or their combination in the presence or absence of the peptide caspase inhibitor ZVAD-fmk. Caspase inhibition significantly reversed the toxicity of TRAIL alone, doxorubicin alone, and the combination of TRAIL and doxorubicin (Fig. 7 top panel). This suggested that caspase activation by doxorubicin might mediate the augmentation of TRAIL-induced apoptosis by doxorubicin.

The ability of ZVAD-fmk to reverse toxicity of each chemotherapy agent in MDA-MB-453 cells was then analyzed. Caspase inhibition significantly inhibited the toxicity of doxorubicin and 5-fluorouracil

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**Table 1** Augmentation of TRAIL toxicity by chemotherapy in breast cell lines

<table>
<thead>
<tr>
<th>TRAIL + Chemotherapy</th>
<th>Normal breast cell lines</th>
<th>Malignant breast cell lines</th>
<th>Total breast cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL + doxorubicin 5 μM</td>
<td>4/4</td>
<td>6/7</td>
<td>10/11</td>
</tr>
<tr>
<td>TRAIL + 5-fluorouracil 30 μM</td>
<td>4/4</td>
<td>4/6</td>
<td>8/10</td>
</tr>
<tr>
<td>TRAIL + paclitaxel 5 μM</td>
<td>1/4</td>
<td>0/6</td>
<td>1/10</td>
</tr>
<tr>
<td>TRAIL + melphelan 100 μM</td>
<td>3/4</td>
<td>1/6</td>
<td>4/10</td>
</tr>
<tr>
<td>TRAIL + methotrexate 500 μM</td>
<td>0/3</td>
<td>0/6</td>
<td>0/9</td>
</tr>
</tbody>
</table>

*Data represent the number of cell lines where there is significant augmentation of TRAIL-mediated toxicity by chemotherapy agents/total number of cell lines analyzed. Results were considered significant if the toxicity of the combination of TRAIL and the chemotherapy agent by fractional inhibition analysis was synergistic compared with TRAIL alone or the chemotherapy agent alone.*
DOXORUBICIN

METHOTREXATE

TRAIL AND BREAST CELL LINES

TRAIL compared with TRAIL alone. To demonstrate that caspase-3 and PARP cleavage were due to apoptosis and not to nonspecific proteolysis, expression of ERK-2 was analyzed. ERK-2 is not cleaved during apoptosis in many systems (42), and it is also not cleaved in MDA-MB-453 cells incubated with TRAIL, doxorubicin, or their combination (Fig. 8).

DISCUSSION

To investigate induction of apoptosis by TRAIL, several constructs of the TRAIL extracellular domain were produced as GST fusion proteins (Fig. 1). Of the TNF family, TRAIL is most homologous to Fas ligand (23), and these constructs were selected by homology with TNF family members including Fas ligand. Two regions of the Fas ligand extracellular domain are required to induce apoptosis, an extreme COOH-terminal receptor binding domain and a more NH₂ located self-association domain. Interestingly, constructs with deletion of TRAIL COOH-terminal regions (amino acids 95–229 and 95–191) homologous to the Fas ligand receptor binding domain were incapable of inducing apoptosis (43). Also, TRAIL constructs that deleted part or all (amino acids 153–281 and 203–281) of the regions homologous to the Fas ligand self-association domain did not induce apoptosis (43). This suggests that activation of death receptors by TRAIL requires similar structural and functional characteristics to those of Fas ligand.

TRAIL induced significant apoptosis in only 2 of 16 (12.5%) breast cell lines (Fig. 2). This is consistent with the finding that a minority (27%) of tumors of hematological origin are sensitive to TRAIL-induced apoptosis (44). The spectrum of breast cell lines sensitive to TRAIL-induced apoptosis (MCF10A and MDA-MB-231) also differed from those we demonstrated previously to be sensitive to Fas-induced apoptosis (HMEC 2595, MCF10A, 184B5, and T47D; Ref. 4). Overlapping but different sensitivities to Fas and TRAIL-induced apoptosis have also been demonstrated in hematological malignancies (44) and in lymphocyte subsets (25, 45).

Consistent with data from other systems was expression of the activating TRAIL death receptors DR4 and DR5 in all breast cell lines analyzed (Fig. 3; Refs. 15–17). However, in contrast with published data, expression of the inhibitory receptor, TRID was not restricted to nonmalignant cell lines (16, 17, 27). In fact, TRID was highly expressed in breast cancer cell lines (two of seven) as frequently as in normal breast cell lines (one of four). TRID expression also did not predict for sensitivity to TRAIL-induced apoptosis in breast cells. TRAIL induced significant apoptosis in breast cell lines that expressed high levels (MDA-MB-231) and low levels (MCF10A) of TRID. Notably, the relative expression of the inhibitory receptor, TRID was not restricted to breast cancer cell lines (two of seven) as frequently as in nonmalignant cell lines (16, 17, 27). In fact, TRID was highly expressed in breast cancer cell lines (two of seven) as frequently as in normal breast cell lines (one of four). TRID expression also did not predict for sensitivity to TRAIL-induced apoptosis in breast cells. TRAIL induced significant apoptosis in breast cell lines that expressed high levels (MDA-MB-231) and low levels (MCF10A) of TRID. Notably, the relative expression of the activating death receptors DR4 and DR5 was higher in the responsive MDA-MB-231 cell but not of melphalan, paclitaxel, and methotrexate (Fig. 7, bottom panel). This further suggested that those chemotherapy agents that sensitized cells to TRAIL-induced apoptosis also activated caspases in MDA-MB-453 cells.

To directly assess activation of caspases, cleavage of caspase-3 and PARP was analyzed in TRAIL-resistant MDA-MB-453 cells incubated with TRAIL alone and in combination with doxorubicin or methotrexate (Fig. 8). Caspase-3 is activated by cleavage from a M₀ 32,000 precursor into M₁ 17,000 and M₂ 11,000 products (39). Activated caspase-3 cleaves PARP from a M₀ 116,000 protein into M₁ 85,000 and M₂ 25,000 cleavage products (40, 41). Caspase-3 and PARP are partially cleaved in cells treated with doxorubicin alone. There is also very minimal cleavage of caspase-3 and PARP in cells treated with TRAIL alone. The combination of TRAIL and doxorubicin results in significantly greater cleavage of caspase-3 and PARP than in cells treated with either agent alone. In contrast, methotrexate causes little cleavage of caspase-3 or PARP. Furthermore, there is no increase in the amount of caspase-3 or PARP cleavage in cells incubated with the combination of methotrexate and 5 mM means for quadruplicate results from a representative experiment; bars, SD.

![Image](cancerres.aacrjournals.org)
achieved in patients (38). In contrast, the concentrations of 5-fluorouracil (3–30 μM) that sensitized cells to TRAIL-induced apoptosis were >100-fold over the clinically relevant range (38). Clinically relevant doses of 5-fluorouracil (1–10 μM) did not sensitize breast cells to TRAIL-induced apoptosis. Doxorubicin therefore may be more useful for in vivo studies in combination with TRAIL. Of note, doxorubicin equally sensitized normal and malignant breast cell lines to TRAIL-induced apoptosis. This finding may have particular clinical relevance because other normal tissues typically affected by doxorubicin, e.g., bone marrow, gastrointestinal mucosa, and cardiac tissue, may also be sensitized to TRAIL-induced apoptosis by doxorubicin. Future in vivo studies of chemotherapy and TRAIL will address the issues of combined toxicities to normal tissues and efficacy in treating breast cancers as compared with cell lines.

These data show that chemotherapy agents that themselves activate caspases (doxorubicin and 5-fluorouracil) also augment TRAIL-induced apoptosis (Figs. 7 and 8). In contrast, chemotherapy agents that do not activate caspases also do not augment TRAIL-induced apoptosis. The mechanism of sensitization of breast cell lines to TRAIL-induced apoptosis thus appears to be mediated through caspase activation. Chemotherapy, particularly anthracyclines, have been demonstrated to activate caspases through a variety of mechanisms (12, 30, 51–55). Similarly, signaling through DR4 and DR5 activates caspases (15–17, 25). The significant augmentation of caspase activation seen with the combination of TRAIL and doxorubicin (Fig. 8) may be due to amplification of caspase cleavage upon reception of two independent activating signals. This finding has therapeutic implications because it suggests that relative resistance to apoptosis may be overcome by combining agents that independently partially activate caspases (51). The exact mechanism of this augmentation of caspase activation is not yet known.

In summary, these data demonstrate no difference in expression of Fas family members or in sensitivity to TRAIL-induced apoptosis between normal and malignant breast cancer cell lines, in contrast to results published previously (16, 17). They also demonstrate that

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**Fig. 7.** Caspase inhibition reverses toxicity of chemotherapy agents that augment TRAIL-induced apoptosis. *Top panel,* MDA-MB-453 cells were incubated with doxorubicin, TRAIL, and TRAIL + doxorubicin for 16 h in the absence (■) or presence (●) of ZVAD-fmk (50 μM). *Bottom panel,* MDA-MB-453 cells were incubated for 16 h in the absence (■) or the presence (●) of ZVAD-fmk (50 μM) and the indicated chemotherapy agents. Viability was assessed by MTT assay, and viability results are given as a percentage of control cells. Data points show the averages for quadruplicate results from a representative experiment. *bars,* 5-FU, 5-fluorouracil; MTX, methotrexate. Ps for the difference between treated and untreated cells are shown above bar graphs.

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**Fig. 8.** Western blot analysis of caspase-3, PARP, and ERK-2 expression in MDA-MB-453 cells treated for 16 h with TRAIL, doxorubicin or methotrexate, and the combination of doxorubicin or methotrexate and TRAIL. *Upper panel,* uncleaved caspase-3 (p32) and the caspase-3 cleavage product (p17). *Middle panel,* uncleaved PARP (p116) and cleaved PARP (p85). *Lower panel,* no cleavage of ERK-2 (p42).
chemotherapy can significantly enhance sensitivity to TRAIL-induced apoptosis in breast cell lines, and this enhancement is mediated through caspase activation. These data suggest that activation of the TRAIL pathway in combination with other agents that activate caspases in breast cancer cells may have therapeutic potential in treatment of breast cancer.

REFERENCES


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