

Tumor Necrosis Factor–Related Apoptosis-Inducing Ligand and Chemotherapy Cooperate to Induce Apoptosis in Mesothelioma Cell Lines

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Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) can induce apoptosis in certain tumor cells. In addition, TRAIL and chemotherapy can act cooperatively, possibly as a result of chemotherapy-induced increases in expression of a TRAIL receptor, DR5. We used cell lines derived from a highly chemoresistant tumor, malignant mesothelioma, to learn whether TRAIL was effective alone or together with chemotherapy and whether cooperativity depended on increases in DR5 expression. TRAIL (codons 95–285) was expressed in a bacterial expression vector and purified by nickel affinity chromatography. TRAIL alone (25 to 500 ng/ml) had little effect on mesothelioma cells. TRAIL plus chemotherapy (doxorubicin, cis-platinum, etoposide, or gemcitabine) acted cooperatively to induce apoptosis in mesothelioma cells (M28, REN, VAMT, and MS-1). For example, in M28 cells treated for 18 h, apoptosis from TRAIL (100 ng/ml) plus doxorubicin (0.6 μ g/ml; $71 \pm 11\%$) greatly exceeded that from TRAIL alone ($21 \pm 8\%$) or from doxorubicin alone ($6 \pm 2\%$) (means \pm standard deviation; $P < 0.03$). Mesothelioma cells treated with chemotherapy showed no change in DR5 protein by Western analysis or by immunocytochemistry. TRAIL plus chemotherapy was associated with an increase in mitochondrial cytochrome c release and mitochondrial depolarization. We conclude that TRAIL and chemotherapy act cooperatively to kill mesothelioma cell lines, not by increases in DR5 receptor but in association with mitochondrial amplification of apoptotic signals.

Mesothelioma is a highly chemoresistant tumor. In separate studies, mesothelioma has shown less than 20% response rates to single chemotherapeutic agents (1). The lack of response may relate in part to a resistance of the tumor to apoptosis (2). Indeed, mesothelioma cell lines have shown a high level of resistance to apoptosis from a variety of apoptotic stimuli, although the mechanism of the resistance to apoptosis has not been determined (3). For example, we have shown that Bcl-2 overexpression is not a feature of mesothelioma cell lines (3). Multiple studies have suggested that p53 mutations are rare in mesothelioma (4, 5), although the p53 pathway may be impaired by abnormalities in other proteins, such as p14 (6). Apoptosis is now understood to involve two major signaling pathways, one initiated by DNA damage and one initiated by death receptors (DRs). Resistance to chemotherapeutic agents may be due to an impairment in the DNA damage pathway to apoptosis; if so,

resistance may possibly be bypassed by activation of the death receptor-mediated pathway.

One death ligand, tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) or Apo-2L, has shown an ability to kill cancer cell lines both *in vitro* and *in vivo* (7, 8). One attractive feature of TRAIL for possible clinical application has been the resistance of many normal cell lines to TRAIL, with the recently discovered exception of cultured human hepatocytes (9). This ligand initiates a death receptor pathway to apoptosis; analogous to the fas ligand, TRAIL engages its receptor, DR5 or DR4, and recruits an adaptor protein, Fas-associated death domain (FADD), that recruits and induces activation of the initiator caspase, caspase 8 (10, 11). When used in combination with agents that likely induce a DNA-damage pathway to apoptosis, such as chemotherapy or irradiation, TRAIL has demonstrated increased ability to induce apoptosis (12–17). It appears that activation of the two separate pathways to apoptosis can amplify the apoptotic response, although the mechanism is not understood. Synergy between death receptor and DNA-damage apoptotic pathways has also been described for fas ligand, often attributed to the upregulation of the fas receptor (18, 19). Similarly, in the case of TRAIL, the mechanism of the synergy has been proposed to be via the upregulation of the TRAIL receptor DR5 (15–17).

We wished to test the effect of TRAIL on mesothelioma cell lines, both with and without chemotherapy, to determine whether cooperativity could be identified and, if so, whether the enhanced apoptosis could be explained by changes in DR5 expression. We found that TRAIL plus chemotherapy act cooperatively to kill mesothelioma lines and that the cooperation does not involve changes in DR5 expression. Instead, we found that the cooperation was associated with amplification of mitochondrial release of cytochrome c and mitochondrial depolarization.

Materials and Methods

Reagents and Proteins

TRAIL codons 95–285 were amplified by polymerase chain reaction (PCR) and subcloned into a pQE-9 (Qiagen, Santa Cruz, CA) bacterial expression vector downstream of a 6X-Histidine tag. TRAIL was purified by nickel affinity chromatography according to the manufacturer's protocol and 10 μ M ZnCl₂ was added to maintain the trimeric structure. The purity of TRAIL was confirmed by Coomassie blue staining.

Inhibitors of caspases zVAD-fmk (a pan-caspase inhibitor) and zLEHD-fmk (a caspase 9 inhibitor), and TNF- α were purchased from R&D Systems, Inc. (Minneapolis, MN). Crosslinking anti-Fas IgM monoclonal antibody (mAb) (CH-11) was purchased from Medical & Biological Laboratories (Nagoya, Japan). Chemotherapeutic agents used included doxorubicin and etoposide.

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Abbreviations: cis-platinum, CDDP; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; propidium iodide, PI; standard deviation of the mean, SD; tumor necrosis factor, TNF; TNF-related apoptosis-inducing ligand, TRAIL; *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone, zVAD-fmk.

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side (Bedford Laboratories, Bedford, OH), cis-platinum (CDDP) (Sigma, St. Louis, MO), and gemcitabine (Eli Lilly and Co., Indianapolis, IN). Concentrations were selected as those that decreased viability of the cells to between 70 and 80% of control: doxorubicin (0.6 $\mu\text{g/ml}$; 1 μM), CDDP (10 $\mu\text{g/ml}$; 33 μM), etoposide (15 $\mu\text{g/ml}$; 25 μM), and gemcitabine (0.3 $\mu\text{g/ml}$; 1 μM). The concentrations of chemotherapeutic agents chosen are consistent with clinically relevant doses (20, 21).

Cell Lines

Human mesothelioma lines used were: M28 and VAMT1 (both with wild-type p53), obtained from Dr. Brenda Gerwin (3, 4), National Cancer Institute (Bethesda, MD), REN and LRK1A (both with abnormal p53), from Dr. Steven Albelda, University of Pennsylvania, Philadelphia; and MS-1 from Dr. Steven Idell, University of Texas at Tyler (Tyler, TX). HeLa cells were obtained from American Type Culture Collection (Manassas, VA) and used as a positive control for expression of DR5 (22). Normal human mesothelial cells were harvested from benign pleural effusions or ascites as described (23).

Cells and Culture

Tumor cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)/RPMI 1640 (1:1) supplemented with *N*-1-hydroxyethylpiperazine-*N'*-ethane sulfonic acid (Hepes) (10 mM), 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), L-glutamine (2 mM; GIBCO BRL, Grand Island, NY), penicillin (100 U/ml; GIBCO), and streptomycin (100 $\mu\text{g/ml}$; GIBCO). The primary cultured human cells were grown in LHC-MM medium (Biofluids, Rockville, MD), L-glutamax II (2 mM; GIBCO), penicillin, and streptomycin.

Assay for Viability

Cellular metabolism was detected by the reduction of alamarBlue dye and its change from an oxidized (nonfluorescent blue) to a reduced (fluorescent red) form. After experiments involving cells in 24-well plates for 18 h, media were removed and replaced with fresh media containing 10% alamarBlue (Accumed International, Westlake, OH) for a 2-h incubation in a 37°C 5% CO₂ incubator. After incubation, supernatant from each well was transferred to four wells of a 96-well plate and the fluorescence was determined in quadruplicate with excitation at 530 nm and emission detected at 590 nm using a microplate reader (Wallac Victor multilabel counter; EG&G WALLAC, Wallac, Oy, Turku, Finland). Viability was expressed as the fluorescence (minus background) in supernatant from treated cells as a percentage of the fluorescence (minus background) in supernatant from control, untreated cells.

Apoptosis Assays

Annexin V assay for apoptosis. Apoptosis was measured by the binding of green fluorescent protein (GFP)-annexin V to the phosphatidylserine residues on the outer leaflet of the apoptotic cellular membrane, using a GFP-annexin V fusion protein we constructed (24).

The cell pellet was resuspended in serum-free RPMI/DMEM buffer and stained with GFP-annexin V fusion protein (3 $\mu\text{g/ml}$ in Hepes buffer) for 10 min on ice, as described (25). Propidium iodide (PI) (15 $\mu\text{g/ml}$) was added just before analysis by flow cytometry. Because necrosis was not detected morphologically in these cells over the course of these experiments (*see later text*), all annexin V-positive cells were considered apoptotic: PI-negative cells as early apoptotic, PI-positive cells as late apoptotic. Cells were analyzed using a FAC-Scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ), with acquisition and data analysis as described earlier. A total of 10,000 events per sample were acquired to ensure adequate mean data.

Morphologic assay using acridine orange staining. Cells stained with acridine orange and PI were examined for morphologic evi-

dence of apoptosis, as described (25). Apoptosis, both early (PI⁻) and late (PI⁺), was identified by the presence of condensed, highly fluorescent nuclei. Necrosis, as identified by PI⁺ cells with a lack of nuclear condensation, was seen in fewer than 3% of cells in all experiments.

Western analysis for expression of DR5 protein. Lysates were prepared on ice from 15-cm plates of M28, REN, LRK1A, HeLa, or primary human mesothelial cells after incubation with doxorubicin (0.6 $\mu\text{g/ml}$) or control media for 18 h. The lysis buffer consisted of 1% Triton X100, 0.1% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride in cell wash buffer (10 mM Tris [pH 7.4], 150 mM NaCl, and 0.025% NaN₃). Protein concentrations were determined by chromogenic microplate assay using BCA Protein Assay Reagent (Pierce, Rockford, IL).

Samples (50 $\mu\text{g/lane}$) were boiled for 5 min with sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% nonfat milk (in PBS plus 0.05% Tween-20) for 1 h at room temperature. Blots were incubated overnight at 4°C with rabbit antihuman DR5 polyclonal antibody (Chemicon, lot #20060415,) diluted 1:500 in blocking solution. The membrane was washed in PBST five times for 12 min each, then incubated with 1:1,000 horseradish peroxidase (HRP)-conjugated antirabbit IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. After washing, blots were developed using ECL reagent (Amersham) and autoradiography film. Molecular weights were calculated by comparison with Biorad Kaleidoscope markers.

Reverse transcriptase (RT)-PCR for DR5 and DR4 message. Floating and attached cells were harvested together from 15-cm plates of M28 cells after incubation for 8 or 24 h with doxorubicin (0.6 $\mu\text{g/ml}$), CDDP (10 $\mu\text{g/ml}$), TRAIL (100 ng/ml), or normal media. Messenger RNA was extracted from the cells using the Nucleospin II Kit (Clontech, Palo Alto, CA). Yield and purity of the RNA samples were determined by A_{260/280nm} spectrometry. In both reverse transcription and PCR steps, all reaction reagents were prepared as master mixes and then aliquotted. Reverse transcription was performed on 2 μg of total RNA for each sample. PCR was performed under the following conditions: 30 cycles with denaturation at 94°C for 30 s, annealing at 68°C for 4 min, and extension at 68°C for 1 min. The primers for DR4 were upstream 5'-CTTCAAGTTTGTCTCGTCGTCG-3' and downstream 5'-GAGCCGATGCAACAACAGAC-3'; and for DR5, upstream 5'-ATCACCAACAAGACCTAGC-3' and downstream 5'-TCC-TCAATCTTCTGCTTGGC-3'.

Immunocytochemistry for DR5 distribution. Cells (10⁴) were plated overnight on each of three uncoated wells of Teflon-coated slides (Structure Probes, Inc., Westchester, PA). Doxorubicin (0.6 $\mu\text{g/ml}$) was added for measured time periods up to 16 h. Cells were washed in PBS, fixed in -20°C methanol for 5 min, and washed again. Cells were blocked in 10% normal rabbit serum diluted in PBT (PBS plus 0.2% Triton-X100) for 30 min at room temperature. Cells were then incubated with goat antihuman DR5 (Calbiochem, San Diego, CA; 1:100) in 1.5% normal rabbit serum in PBT overnight at 4°C. As a control, cells were incubated with an irrelevant primary antibody, goat antihuman presenilin-1 (Calbiochem; 1:100). After three washes in PBT and one wash in PBS, cells were incubated with biotinylated rabbit antigoat IgG antibody (Vector Labs, Burlingame, CA; 1:250) for 2 h at room temperature, washed, and then incubated with fluorescein isothiocyanate-labeled streptavidin (Vector Labs; 1:250) for 2 h at room temperature. Nuclei were counterstained with DAPI (Molecular Probes, Inc., Eugene, OR; 0.1 $\mu\text{g/ml}$) for 10 min. Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

Assay of cytoplasmic cytochrome C release. M28 cells on 10- to 15-cm plates at 80% confluence were washed with cold PBS, harvested by gently scraping in ice-cold PBS on ice, and pelleted by

centrifugation. After a wash with PBS, the cell pellet was quickly resuspended in ice-cold HEB (20 mM Pipes [pH 7.4], 10 mM KCl, 5 mM EDTA, and 2 mM MgCl₂, with 2 mM dithiothreitol added before use) and pelleted by centrifugation. The cell pellet was resuspended in 300 μ l of HEB and allowed to swell on ice for 30 min. To disrupt the cells while leaving the mitochondria intact, cells were passed through a 25-gauge needle five times. The lysates were pelleted by centrifugation at full speed in a benchtop microcentrifuge for 30 min at 4°C. The resulting supernatant was collected as cell extracts and stored at -80°C. Protein (50 μ g) from the cell extracts was run on 15% SDS-PAGE (National Diagnostics, Atlanta, GA) and transferred to a PVDF membrane (Millipore Corp., Bedford, MA). The membrane was incubated with anti-cytochrome c mAb (PharMingen, San Diego, CA; 1:500) followed by a secondary HRP-labeled sheep antimouse antibody (Amersham Pharmacia Biotech; 1:3,000). Signals were detected by ECL reagent (Amersham Pharmacia Biotech) and quantified by densitometry using NIH image (<http://rsb.info.nih.gov/nih-image/>).

Mitochondria depolarization assay. Loss of the mitochondrial potential difference was measured using ApoAlert Mitochondrial Membrane Sensor Kit (Clontech). M28 cells were trypsinized from 24-well plates and pelleted by centrifugation. The cells were loaded with the mitochondrial sensor dye by resuspending the cell pellets in 200 μ l MitoSensor reagent (1 μ l MitoSensor in 1 ml incu-

bation buffer; final concentration 5 μ g/ml) and incubating them at 37°C in 5% CO₂ for 20 min. The cells were washed and resuspended in incubation buffer, and analyzed by flow cytometry. In cells with normal mitochondria, the dye aggregates in the mitochondria, fluoresces red, and is detected in the FL2 channel. However, when mitochondrial membrane potentials are altered, the dye cannot accumulate in the mitochondria, remains as monomers in the cytoplasm, fluoresces green, and is detected in the FL1 channel. The degree of mitochondrial depolarization was calculated as the percentage of cells shifting to a region defined as a higher FL1 and lower forward scatter (FSC), as described (26).

Statistics

Data were analyzed by analysis of variance with *post hoc* analysis by Tukey's test (InStat 2.0; GraphPad Software, Inc., San Diego, CA). Data are described as means \pm standard deviation of the mean (SD) unless otherwise noted. A difference was regarded as significant if $P < 0.05$.

Results

Effect of TRAIL on Viability

Two mesothelioma cell lines (M28 and REN) were exposed to TRAIL alone or TRAIL plus chemotherapy for 18 h (Figure 1). The effect of TRAIL was compared with that of TNF, another DR ligand. We used the doses of CDDP and doxorubicin that caused a reduction of viability to approx-

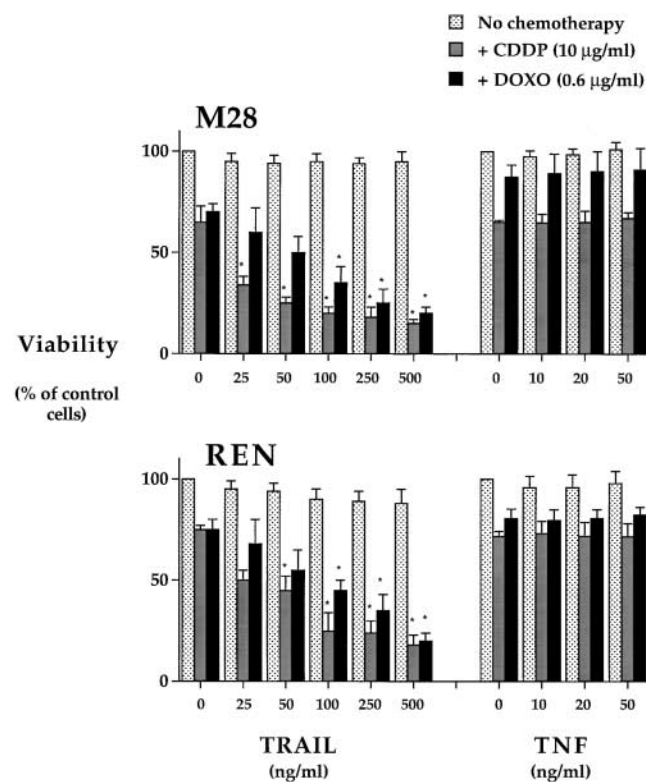


Figure 1. Loss of viability of mesothelioma cell lines after TRAIL plus chemotherapy. Two mesothelioma cell lines, M28 and REN, were cultured for 18 h with TRAIL (25 to 500 ng/ml) or TNF (10 to 50 ng/ml) with or without CDDP (10 μ g/ml) or doxorubicin (DOXO; 0.6 μ g/ml). The doses of chemotherapy were chosen as those that reduced viability of treated cells to between 70 and 80% of control, untreated cells. Viability was determined by the reduction of alamarBlue dye as a percentage of control, untreated cells. * indicates more toxicity from the combined treatment than from the sum of toxicities of TRAIL and chemotherapy alone ($n = 3$ experiments, read in quadruplicate; means \pm SD; $P < 0.05$).

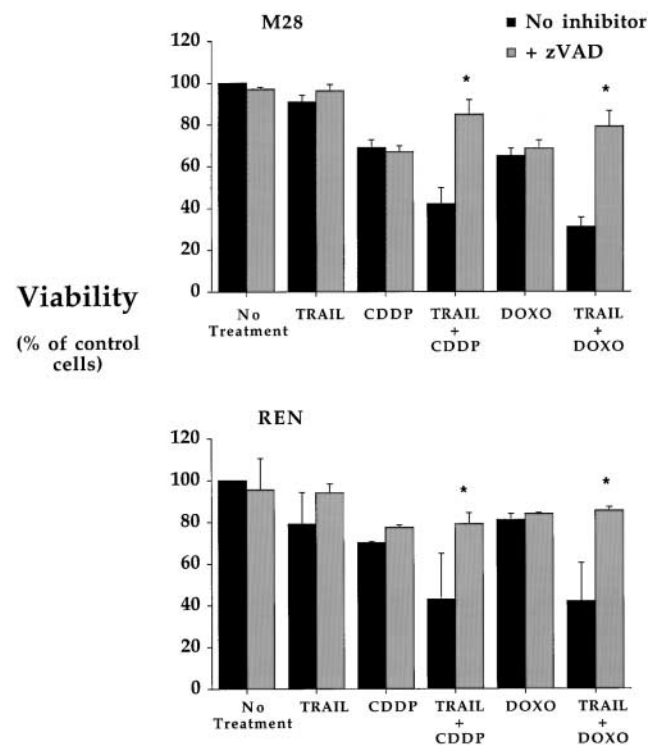


Figure 2. Dependence of cooperative killing on caspases. The M28 and REN cell lines were incubated with no treatment or with TRAIL (100 ng/ml), CDDP (10 μ g/ml), TRAIL plus CDDP, doxorubicin (DOXO; 0.6 μ g/ml), or TRAIL plus doxorubicin for 18 h with or without zVAD-fmk (40 μ M), a pan-caspase inhibitor. The decrease in viability induced by the combination of TRAIL plus chemotherapy was blocked by zVAD. ($n = 5$; means \pm SD; $P \leq 0.05$ different from the same condition without zVAD).

imately 75% of the control cells (10 and 0.6 $\mu\text{g/ml}$, respectively). TRAIL (25 to 500 ng/ml) and TNF (0 to 50 ng/ml) alone had little effect on the cell lines. TRAIL, unlike TNF, was effective in combination with chemotherapy, inducing a significantly greater reduction in viability than either TRAIL or chemotherapy alone. Anti-fas (CH-11, 0 to 1,000 ng/ml) alone or together with chemotherapy similarly had no effect on the cell lines, but the two cell lines expressed low levels of fas receptor by flow cytometry

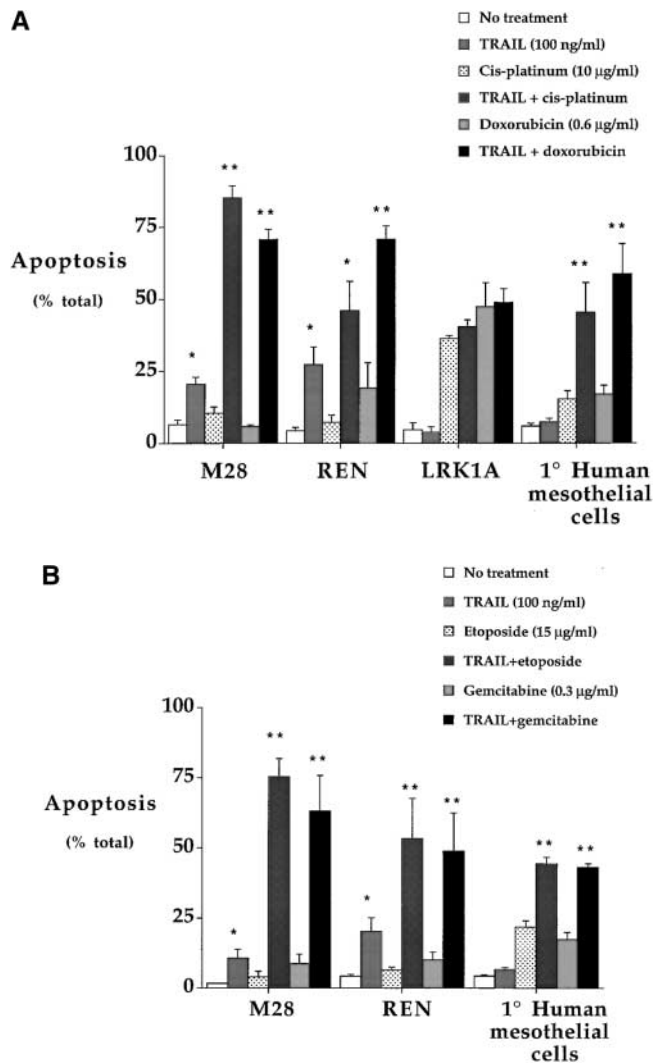


Figure 3. Induction of apoptosis by TRAIL plus chemotherapy. Mesothelioma cell lines and primary human mesothelial cells were exposed to TRAIL (100 ng/ml) alone, chemotherapeutic agents alone, or combinations of TRAIL plus chemotherapy for 18 h and assayed for apoptosis by annexin V staining. TRAIL alone had no effect on LRK1A or primary mesothelial cells. Compared with each treatment alone, TRAIL plus chemotherapy significantly increased apoptosis in all cells except LRK1A. (A) M28, REN, LRK1A, and primary cells were exposed to TRAIL, CDDP (10 $\mu\text{g/ml}$), doxorubicin (0.6 $\mu\text{g/ml}$), or a combination. (B) M28, REN, and primary cells were exposed to TRAIL, etoposide (15 $\mu\text{g/ml}$), gemcitabine (0.3 $\mu\text{g/ml}$), or a combination. ($n = 5$ experiments; means \pm SD; * $P < 0.05$ different from no treatment; * $P < 0.05$ different from the sum of apoptosis due to TRAIL alone plus chemotherapy alone.)

(data not shown). TNF responsiveness of the cell lines was confirmed by the ability of actinomycin D to enhance TNF-induced apoptosis (data not shown). For all later studies, TRAIL was used at 100 ng/ml.

The role of caspase activation was studied by addition of zVAD, a pan-caspase inhibitor, 1 h before the TRAIL and/or chemotherapy (Figure 2). For both cell lines, zVAD (40 μM) significantly inhibited the cooperative effect of TRAIL plus chemotherapy on viability.

Induction of Apoptosis by TRAIL with and without Chemotherapy

To confirm whether the loss of viability was due to apoptosis, we measured apoptosis in cell lines and normal human mesothelial cells exposed to TRAIL and/or chemotherapy for 18 h (Figure 3). TRAIL alone induced some apoptosis in mesothelioma lines M28 and REN. It had no effect on LRK1A or on primary human mesothelial cells. Together with chemotherapy, however, the effect of TRAIL was greatly amplified for M28, REN, and primary human cells. LRK1A, however, remained resistant to TRAIL, showing no effect greater than with chemotherapy alone. Apoptosis was confirmed using morphologic assays for nuclear condensation; in M28 cells treated for 18 h, apoptosis was also greater after TRAIL plus chemotherapy (TRAIL plus CDDP, $23 \pm 7\%$; TRAIL plus doxorubicin, $32 \pm 5\%$) than after TRAIL alone (100 ng/ml; $8 \pm 5\%$) or chemotherapy alone (CDDP, 10 $\mu\text{g/ml}$, $2.5 \pm 3\%$; doxorubicin, 0.6 $\mu\text{g/ml}$, $2 \pm 3\%$) (means \pm SD; $n = 3$; $P \leq 0.04$).

In all TRAIL-sensitive cells, M28, REN, and normal human mesothelial cells, apoptosis could be completely inhibited by inhibition of caspases with zVAD (shown for M28 in Figure 4). The cooperative effect of TRAIL and chemotherapy was not confined to these cell lines, but was similarly found with other mesothelioma cell lines (VAMT and MS-1; data not shown).

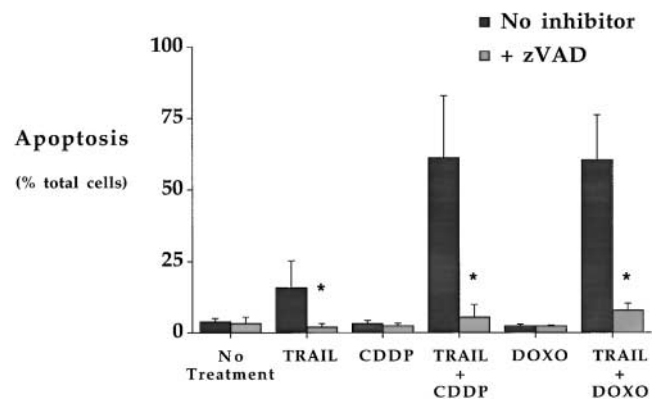


Figure 4. Dependence of apoptosis induced by TRAIL plus chemotherapy on caspases. M28 cells were exposed to TRAIL (100 ng/ml), CDDP (10 $\mu\text{g/ml}$), doxorubicin (DOXO; 0.6 $\mu\text{g/ml}$), or a combination of TRAIL plus chemotherapy for 18 h either with or without zVAD-fmk (40 μM) given 1 h earlier. Inhibition of caspases with zVAD completely blocked the effect of TRAIL and that of TRAIL plus chemotherapy. ($n = 3$ experiments, means \pm SD; * $P < 0.05$ different from the same condition without zVAD.)

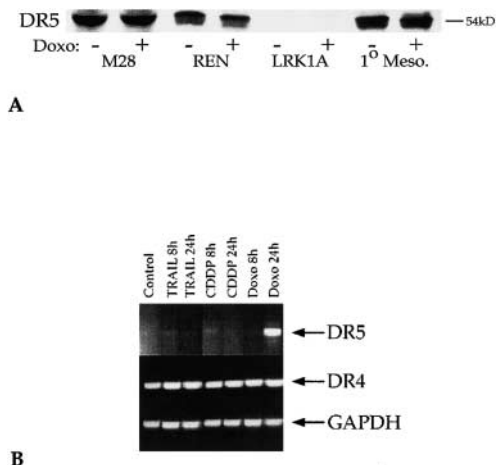


Figure 5. Expression of DR5 protein and DR5 message before and after doxorubicin treatment. (A) M28, REN, and primary human mesothelial cells expressed DR5 protein whereas LRK1A, a line with no response to TRAIL, had no DR5 expression. After exposure to doxorubicin (0.6 $\mu\text{g/ml}$) for 18 h, no cell line showed a change in DR5 protein expression. Equal protein loading was confirmed by Ponceau staining. (Representative of three Western blots.) (B) M28 cells were incubated with TRAIL (100 ng/ml), CDDP (10 $\mu\text{g/ml}$) or doxorubicin (Doxo; 0.6 $\mu\text{g/ml}$) for 8 and 24 h and harvested for RT-PCR (see MATERIALS AND METHODS). Transcripts of DR5 were elevated only in one condition, exposure to doxorubicin at 24 h. (Experiment shown is representative of three.)

Expression of DR5 Protein and Effect of Chemotherapy on Expression of DR5 Protein and Message

By Western analysis, M28, REN, and primary mesothelial cells expressed DR5 protein whereas LRK1A cells, the cell line resistant to TRAIL, showed no DR5 protein (Figure 5A). As a positive control, HeLa cells also expressed DR5 (not shown). Both mesothelioma and normal mesothelial cells exposed to doxorubicin for 18 h showed no increase in DR5 protein (Figure 5A).

By RT-PCR, M28 cells exposed to doxorubicin or CDDP for 8 h showed no change in message for DR5 or for DR4 (Figure 5B). After exposure to these agents for 24 h, message for DR5 was increased in M28 cells exposed to doxorubicin but not to CDDP.

By immunocytochemistry, DR5 was shown to be present in M28, REN, and normal human mesothelial cells but to be absent in LRK1A (Figure 6). DR5 appeared to be located in intracellular perinuclear compartments such as the trans-Golgi network, as has been shown previously (27). After doxorubicin treatment for 1, 4, and 16 h, no visible change in distribution or in intensity of staining could be identified (shown for 16 h in Figure 6).

Effect of TRAIL and Chemotherapy on Cytochrome C Release

To confirm whether the cooperative effect of TRAIL and chemotherapy involved the mitochondria, we measured cytochrome c release. Cytochrome c was detected in the cytoplasm from M28 cells exposed for 18 h to TRAIL alone and to

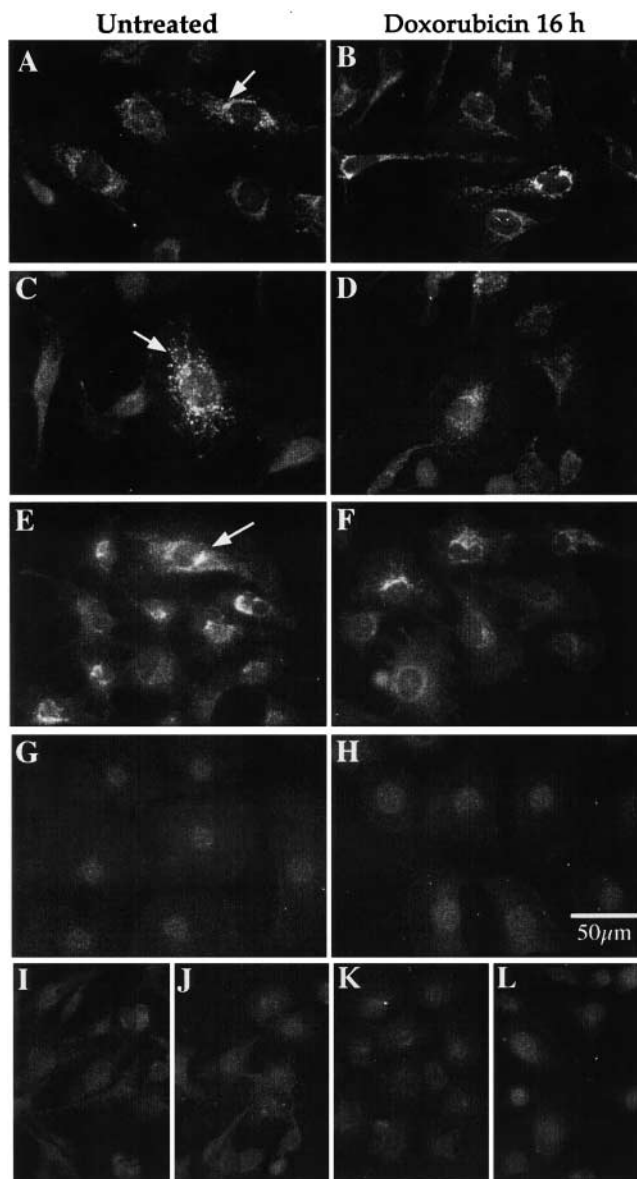


Figure 6. Expression of DR5 protein by immunocytochemistry before or after doxorubicin for 16 h. M28 cells (A and B), REN (C and D), normal mesothelial (E and F) and LRK1A (G and H) cells were exposed to normal media alone (A, C, E, and G) or doxorubicin (0.6 $\mu\text{g/ml}$) (B, D, F, and H) for 16 h, fixed, permeabilized and stained for DR5 (see MATERIALS AND METHODS). DR5 can be seen in a perinuclear location (arrows). There was no evident change in overall expression or distribution of DR5 after doxorubicin. (I–L) Cells were stained with an irrelevant primary antibody (goat antihuman presenilin-1) in M28, REN, normal mesothelial, and LRK1A cells, respectively.

doxorubicin alone (Figure 7A). The cytochrome c release from cells exposed to both agents was increased compared with that due to either agent alone (1.8-fold compared with TRAIL alone; 6.5-fold compared with doxorubicin alone). Because cytochrome c release can be amplified by downstream caspase activation, we repeated these studies with and without inhibition of caspase 9 with zLEHD (40 μM). Despite the inhibition of caspase 9, the increase in cytochrome

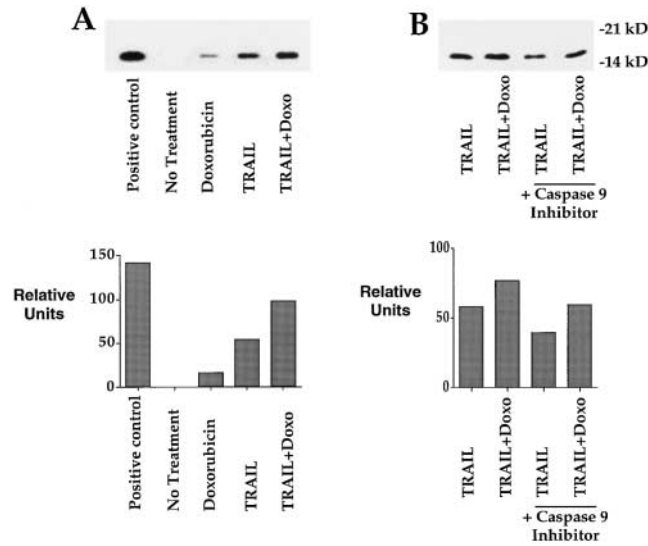


Figure 7. Cytochrome c release after exposure to TRAIL, chemotherapy, or both, and response to inhibition of caspase 9. (A) M28 cells were exposed to no treatment, TRAIL (100 ng/ml), doxorubicin (DOXO; 0.6 μ g/ml), or TRAIL plus doxorubicin for 18 h. A positive control was prepared by extensive cellular disruption to break mitochondrial membranes and release cytochrome c. Cytochrome c was detected by anti-cytochrome c mAb (PharMingen) followed by a secondary HRP-labeled sheep anti-mouse antibody. (B) M28 cells exposed to TRAIL or TRAIL plus doxorubicin for 18 h in presence of zLEHD-fmk, an inhibitor of caspase 9. (Quantitation of blots by densitometry is shown below each blot and expressed in relative units. Results shown are representative of three experiments.)

c release with TRAIL plus doxorubicin compared with TRAIL alone was still seen (Figure 7B).

Effect of TRAIL and Chemotherapy on Mitochondrial Depolarization

As another measure of mitochondrial involvement in the cooperative effect of TRAIL and chemotherapy, we studied mitochondrial depolarization in M28 cells exposed to TRAIL, doxorubicin, or both, as measured by fluorescent shift of cells loaded with a mitochondrial potential-sensitive dye (Figure 8). By 12 h, mitochondrial depolarization showed an enhancement after treatment with both TRAIL and doxorubicin (Figure 8B). This effect corresponded to an enhancement of apoptosis after treatment with both TRAIL and doxorubicin that was also evident by 12 h (Figure 8A). The amplified effect of TRAIL plus chemotherapy on mitochondrial depolarization could be largely blocked by inhibition of caspases with zVAD (100 μ M) (data not shown).

Discussion

In this report, we show that TRAIL has activity in mesothelioma cell lines when used in combination with chemotherapeutic agents. TRAIL greatly enhances the chemosensitivity of the mesothelioma cells to agents used clinically in this disease. The strong cooperative effect involves the caspase system and the mitochondrial apoptotic machinery and is not explained by upregulation of the DR5 receptor for TRAIL. The cooperativity is also not dependent on p53 be-

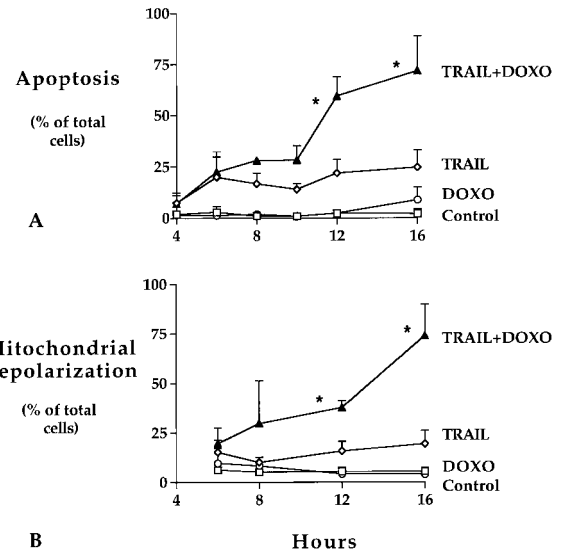


Figure 8. Mitochondrial depolarization and apoptosis of M28 cells after exposure to TRAIL, chemotherapy, or both over 16 h. After exposure to TRAIL (100 ng/ml), doxorubicin (DOXO; 0.6 μ g/ml), or TRAIL plus doxorubicin for different times, M28 cells were harvested. (A) In some experiments, cells were analyzed for apoptosis by staining with annexin V (see MATERIALS AND METHODS). (B) In other experiments, cells were exposed to a mitochondrial sensor dye for 20 min and analyzed by flow cytometry. The percentage of cells moving toward a quadrant of green fluorescence and FSC were quantitated as cells with mitochondrial depolarization (see MATERIALS AND METHODS). ($n = 3$ experiments; means \pm SD; * $P < 0.05$ greater than the sum of effects of TRAIL and doxorubicin alone.)

cause enhancement of apoptosis to TRAIL plus chemotherapy was seen in lines with wild-type p53 (M28 and VAMT) and in a line with mutated p53 (REN). (The cell line that failed to show synergy [LRK1A] was found to lack expression of DR5.) Mesothelioma cells may be amenable to amplification via mitochondria in part because of their low expression of Bcl-2, which could otherwise suppress mitochondrial release of cytochrome c or depolarization (3).

Of the three major death ligands—TNF, fasL, and TRAIL—TRAIL was the most effective at inducing apoptosis in these cells. This may be for several reasons. TNF receptors activate parallel survival pathways, such as nuclear factor (NF)- κ B, to a greater degree than do TRAIL receptors (28). Pre-existing survival factors also apparently interfere with TNF-induced apoptosis, factors that are blocked by inhibition of protein synthesis or transcription (29), as we could show. It is interesting to note that these survival pathways were apparently not interrupted by chemotherapeutic agents, which were not able to sensitize cells to TNF as they clearly did to TRAIL. Fas receptor ligation also had no effect on the cells, but because of the low level of fas receptor expression the lack of response to fas receptor engagement is difficult to interpret. Nonetheless, at least when TRAIL receptors are expressed, TRAIL holds promise for engaging the apoptotic pathway in mesothelioma cells.

TRAIL has shown a relative sparing of normal cells (with the recent exception of normal human hepatocytes [9]). Similarly, in our study, TRAIL alone had no effect on

primary mesothelial cells. Nonetheless, resistance to TRAIL of the primary mesothelial cells was lost when TRAIL was combined with chemotherapy. This suggests that the resistance of these normal cells to TRAIL is relative, perhaps due to a relatively weak TRAIL-induced apoptotic signal that is amplified by concomitant use of chemotherapy. Given systemically, TRAIL, unlike TNF and fas ligand, has been safe in animals (8, 30); however, if TRAIL is ultimately found to be toxic to human hepatocytes *in vivo*, it will limit the clinical use of systemic TRAIL. Malignant mesothelioma, however, is largely confined to the pleural space surrounding the lungs and may be approachable by regional means without risk of liver or other organ injury.

Cooperativity between TRAIL and chemotherapy has been described for other tumor cell lines; in most cases, such enhancement has been ascribed to an upregulation of DR5 message or protein (15–17). In our study, by contrast, there was no evident change in DR5 protein by Western blot or by immunocytochemistry. A strong increase in DR5 message was seen by 24 h after doxorubicin, as could be expected due to the known p53-dependent increases in DR5 transcription (31), but was not accompanied by an increase in protein to explain the observed synergy. It is of note that enhanced killing with TRAIL was also seen with CDDP, an agent that induced no change in DR5 message, even at 24 h (*see* Figure 5). Although we cannot explain the enhanced apoptosis in our cells by changes in DR5 receptor levels, changes in activity or in distribution of the receptor cannot be excluded. However, it appears more likely that, in our cell lines TRAIL and chemotherapeutic agents cooperated via amplification of intracellular apoptotic signals.

It is now recognized that different pathways of apoptotic signals lead to activation of different initiator caspases: the DNA-damage pathway activating caspase 9 and the death receptor pathway activating caspase 8 (26). The DNA-damage signal is sensed in still unknown ways by the mitochondria, inducing a release of cytochrome c to the cytoplasm. Cytochrome c acts together with an adaptor molecule, apoptotic protease activating factor-1, and procaspase 9 to allow autocatalysis and activation of the initiator caspase, caspase 9 (32). The other major pathway is the death receptor pathway in which a death ligand leads to trimerization of a death receptor. As shown for fas receptor and more recently for DR5, the trimerized receptor via its death domains recruits the adaptor molecule, FADD, allowing association and cleavage of procaspase 8 with activation of caspase 8 (10, 11). Once activated by either signaling arm, the initiator caspases 8 and 9 can then activate the distal, executioner caspases, including caspase 3. Although they can operate separately, there are many instances of cross-talk between the two pathways that can lead to amplification of the apoptotic response. The amplification step often includes the contribution of the mitochondria.

Mitochondria may play a key role in amplification of apoptotic signals (32, 33). The fas receptor and more recently DR5 have been shown to induce mitochondrial release of cytochrome c via caspase 8–dependent cleavage of a cytoplasmic molecule, Bid (34, 35). Inclusion of the mitochondrial apoptotic machinery may be necessary, in certain cases, for amplification of weak signals from the death

receptor to generate an apoptotic response (36). The release of cytochrome c from mitochondria can sensitize cells to apoptosis signals originating from the death receptor pathway (37). Caspase-3, activated by the mitochondrial activation of caspase-9, can also activate caspase-8 independent of death receptor involvement, thereby enhancing the apoptosis induced by the chemotherapeutic agent staurosporine (38). Caspase-3, activated by either pathway, can also act on the mitochondria to release more cytochrome c as well as other proapoptotic molecules (apoptosis-inducing factor [AIF], procaspase 3) (38, 39). Mitochondria can thus receive and amplify signals from the death receptor pathway and respond to feedback from downstream caspases.

We identified amplification of mitochondrial release of cytochrome c as associated with the cooperativity between TRAIL and chemotherapy. Both TRAIL and chemotherapy alone induced a release of cytochrome c, with TRAIL possibly interacting with the mitochondria via a DR5-induced Bid cleavage pathway. Each signal alone, however, was not sufficient for apoptosis. When the two were combined, a large apoptotic response was generated. The release of cytochrome c via one signal may have sensitized the cell to the other signal (37) or exceeded a threshold required to activate the downstream caspases (40). Because the increase in release was not blocked by caspase 9 inhibition, we suppose that the amplification is not a result of feedback from mitochondrial activation of downstream caspases but could more likely be explained by cross-talk from the DR pathway at a level proximal to the mitochondria.

Mitochondrial depolarization also was significantly increased by the combination of TRAIL and chemotherapy, supporting a convergence of these pathways at the mitochondria. Depolarization indicates a loss of the mitochondrial inner membrane potential, thought to be caused by the opening of a large membrane channel, the permeability transition pore (PTP), leading to disruption of the outer membrane and release of proapoptotic factors (cytochrome c, procaspase 3, and AIF) (32, 33). The depolarization can be caused by direct stimuli as well as by feedback from distal caspases (40). In our hands, depolarization could be largely blocked by pan-caspase inhibition, suggesting involvement either of proximal caspases such as caspase 8 acting on the mitochondria via molecules such as Bid, or of distal caspases such as caspase 3 known to feedback on the mitochondria to induce PTP opening (39).

In sum, we have shown that TRAIL in concert with chemotherapeutic agents induces apoptosis in otherwise resistant mesothelioma cell lines. In contrast to other reports, we did not observe an increase in DR5 to explain the cooperative effect. We describe an amplification at the level of the mitochondria that suggests cross-talk involving different apoptotic signaling pathways.

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