

Anticancer Agents Sensitize Tumor Cells to Tumor Necrosis Factor-related Apoptosis-inducing Ligand-mediated Caspase-8 Activation and Apoptosis¹

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a new cytokine that was proposed to specifically induce apoptosis of cancer cells. In tumor cells that are resistant to the cytokine, subtoxic concentrations of chemotherapeutic drugs can restore the response to TRAIL. The present study further explores the mechanisms that determine tumor cell sensitivity to TRAIL by comparing four human colon carcinoma cell lines. We show that colon cancer cell sensitivity to TRAIL-induced apoptosis and cytotoxicity correlates with the expression of the death receptors TRAIL-R1 and TRAIL-R2 at the cell surface, as determined by flow cytometry, whereas the two decoy receptors TRAIL-R3 and TRAIL-R4 can be detected only in permeabilized cells. Clinically relevant concentrations of cisplatin and doxorubicin sensitize the most resistant colon cancer cell lines to TRAIL-induced cell death without modifying the expression nor the localization of TRAIL receptors in these cells. TRAIL induces the activation of procaspase-8 and triggers caspase-dependent apoptosis of colon cancer cells. Cytotoxic drugs lower the signaling threshold required for TRAIL-induced procaspase-8 activation. In turn, caspase-8 cleaves Bid, a BH3 domain-containing proapoptotic molecule of the Bcl-2 family, and activates effector caspases. Together, these data indicate that chemotherapeutic drugs sensitize colon tumor cells to TRAIL-mediated caspase-8 activation and apoptosis.

INTRODUCTION

TRAIL³ (1), also known as Apo-2 ligand (2), is a cytokine belonging to the TNF family. Similar to other members of this cytokine family, TRAIL is a type II transmembrane protein with an extracellular COOH-terminal domain (1, 2). This latter domain demonstrated the most significant homology with that of Fas ligand (also known as CD95L or APO-1L; Refs. 1 and 2). Whereas the expression of other members of the TNF family is tightly regulated and limited to activated cells, mRNA of TRAIL is expressed in a wide range of normal tissues including small intestine and colon (1). Similar to most TNF superfamily members, TRAIL can be cleaved at the cell surface to form a soluble molecule (2). Biological studies using either the cell-bound ligand or its soluble form demonstrated the ability of homotrimeric TRAIL to induce apoptosis in transformed cell lines of diverse origin (1, 3). In contrast to Fas-ligand and agonistic anti-Fas mAb, the injection of which causes lethal liver damage to mice (4), injection of a soluble trimerized form of TRAIL did not reveal any toxicity in these animals (5, 6). However, TRAIL was shown recently

to be toxic in isolated human hepatocytes (7) and the normal human brain tissue (8).

The broad expression of TRAIL and its inability to induce apoptosis in normal cells suggested that the regulation of TRAIL-induced cell death was through restricted receptor expression. Two distinct receptors for TRAIL, designated TRAIL-R1 (or DR4; Ref. 9) and TRAIL-R2 (or DR5; Refs. 10–13), were identified. Both are type I transmembrane proteins and contain a death domain similar to that of Fas and TNF-R1 in their cytoplasmic COOH-terminal portions. Their mRNA is expressed in many of the same normal tissues as TRAIL. Two additional receptors for TRAIL designated TRAIL-R3 (or DcR1/TRID; Refs. 10, 11, and 14) and TRAIL-R4 (or DcR2; Refs. 15–17) differ from TRAIL-R1 and TRAIL-R2 by their cytoplasmic domains. TRAIL-R3 is devoid of any transmembrane or cytoplasmic domain and is glycosylphosphatidylinositol-linked to the cell surface, whereas TRAIL-R4 contains only a partial death domain. These two receptors are capable of binding TRAIL with an affinity comparable with that of TRAIL-R1 and TRAIL-R2 (14, 15) but do not mediate apoptosis upon ligation. Thus, TRAIL-R3 and TRAIL-R4 were proposed to act as decoy receptors and to determine whether a cell is resistant or sensitive to TRAIL. In addition, ligation of TRAIL-R4 was suggested to increase the antiapoptotic threshold of cells by activating the transcription factor nuclear factor- κ B (15). However, the expression of TRAIL receptor mRNAs does not correlate with cell sensitivity to TRAIL (3), and the ligation of TRAIL-R1 and TRAIL-R2 also results in the activation of nuclear factor- κ B, although still resulting in apoptosis (12, 18).

Alternative hypotheses for the differential sensitivity of normal and tumor cells to TRAIL include the differential expression of intracellular inhibitors of apoptosis or expression and subcellular location of the death receptors (19). The death signaling pathway through TRAIL-R1 and TRAIL-R2 was shown recently to involve the recruitment of the adaptor molecule FADD in a death-inducing signaling complex (20–22). In turn, FADD recruits procaspase-8 in the death-inducing signaling complex through homophilic interactions between death effector domains contained in the NH₂-terminus of FADD and the prodomain of these caspases (23, 24). Caspase-8 is activated and triggers cell death, either by directly activating downstream effector caspases such as caspase-3 and caspase-6 (25, 26) or by cleaving Bid, a BH3 domain-containing proapoptotic member of the Bcl-2 family of proteins that activates the mitochondrial pathway to cell death (27). These two FADD-dependent cell death pathways can be inhibited by expression of c-FLIP (also called Casper/I-FLICE/FLAME-1/CASH/CLARP/MRIT/Usurpin), a protein with a structure similar to that of caspase-8 but lacking a catalytic site and competitively interacting with FADD, caspase-8, and possibly caspase-10 (28). The expression of c-FLIP was proposed recently to be an essential determinant of cell sensitivity to TRAIL (29).

Several genotoxic agents were reported to up-regulate the expression of TRAIL-R1 and TRAIL-R2 mRNAs by both p53-dependent and independent mechanisms (30–33). This up-regulation was proposed to account for the synergistic activity of TRAIL and chemotherapeutic drugs described in several tumor cell lines (34, 35). In the present study, we compared the sensitivity of four human colon cancer

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³ The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TNF, tumor necrosis factor; mAb, monoclonal antibody; FADD, Fas-associated death domain; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone; z-IETD-fmk, z-Ile-Glu-Thr-Asp-fluoromethylketone; z-DEVD-fmk, z-Asp-Glu-Val-Asp-fluoromethylketone; IETD-AFC, IETD-7-amino-4-tri-fluoromethylcoumarine; DEVD-AMC, DEVD-7-aminomethylcoumarine; PARP, poly(ADP-ribose) polymerase.

cell lines to TRAIL, and we examined the apoptosis sensitization of the most resistant cell lines to soluble TRAIL by the cytotoxic drugs cisplatin and doxorubicin.

MATERIALS AND METHODS

Cell Lines and Cytotoxic Assays. The human colon carcinoma cell lines HT29 (mutated p53), HCT116 (p53 wild-type), and SW480 (mutated p53) were obtained from American Type Culture Collection (Rockville, MD). The HCT8R cell line (p53 wild-type) was kindly provided by Marc Mareel (Ghent, Belgium). These colon cancer cell lines were maintained in Eagle's MEM complemented with 10% FCS (Life Technologies, Inc., Cergy Pontoise, France) and 2 mM L-glutamine (BioWhittaker Co., Fontenay sous Bois, France). For cytotoxic assays, cells (2×10^4) were seeded in 96-well flat-bottomed plates for 24 h and then treated with cytotoxic drugs, soluble human TRAIL (100 ng/ml) cross-linked with 2 μ g/ml of enhancer, or the combination of both for 24 h. Cell viability was determined by the use of a methylene blue colorimetric assay (36), whereas apoptosis was identified by staining nuclear chromatin with 1 μ g/ml Hoechst 33342 for 15 min at 37°C and identifying morphological changes by fluorescence microscopy.

Chemicals and Antibodies. The cytotoxic drugs cisplatin and doxorubicin were obtained from Roger Bellon Chemical Co. (Neuilly, France) and Sigma-Aldrich (Saint-Quentin Fallavier, France), respectively. The caspase inhibitors z-VAD-fmk, z-IETD-fmk, z-DEVD-fmk were from Calbiochem (San Diego, CA). mAbs raised against TRAIL-R1 (M271, IgG2a), TRAIL-R2 (M413, IgG1), TRAIL-R3 (M430, IgG1), and TRAIL-R4 (M445, IgG1) were kindly provided by Dr. M. Kubin (Immunex Corp., Seattle, WA), whereas the blocking mAb RIK2 (IgG1) raised against human TRAIL was a kind gift of Dr. H. Yagita (Juntendo University, Tokyo, Japan). Neutralizing polyclonal goat Abs raised against extracellular domains of TRAIL-R1 and TRAIL-R2 were obtained from R&D Systems (Abingdon, United Kingdom). Mouse IgG1 and IgG2a (Dako Corp., Carpinteria, CA) and goat IgG (Jackson ImmunoResearch Labs, Beckman Coulter, Villepinte, France) were used as isotype-matched controls. The recombinant human soluble TRAIL contains a Flag sequence and can be cross-linked by an enhancer (an antibody anti-Flag); these reagents were obtained from Alexis Biochemicals (Coger, Paris, France). We also used mouse mAbs raised against human procaspase-8 from Immunotech (Coulter, Marseille, France), human HSC70 from Santa Cruz (Tebu, Le Perray en Yvelines, France), Flag (M2) from Sigma-Aldrich, rabbit polyclonal Abs raised against human Bid from R&D Systems, and human Bid from Dr. X. Wang (Howard Hughes Medical Institute, Dallas, TX). All other chemicals were of reagent grade and purchased from local sources.

Flow Cytometry Analyses. The expression of TRAIL-R1 through TRAIL-R4 was studied in the different colon cancer cell lines by flow cytometry after trypsinization. Appropriate concentrations of mAbs were added to the cells in 100 μ l of PBS containing 0.5% BSA (PBS-BSA) and 0.1% sodium azide (Sigma-Aldrich). After 1-h incubation at 4°C and two washes in PBS, cells were incubated for 45 min at 4°C with a FITC-conjugated sheep antimouse IgG (Amersham, Les Ulis, France). These experiments were repeated on cells fixed in 4% paraformaldehyde and permeabilized in saponin (0.1% v/v in PBS-BSA). In all cases, 10,000 cells were analyzed by using a FACScan flow cytometer (Becton Dickinson).

Western Blot Analyses. For immunoblotting, untreated and treated cells were washed in PBS and lysed for 10 min at 4°C in lysis boiling buffer [1% SDS, 10 mM Tris (pH 7.4)] and boiled for 5 min. Proteins (50 μ g) were separated on a polyacrylamide SDS-containing gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Ivry sur Seine, France). After blocking nonspecific binding sites overnight at 4°C by 8% nonfat milk in TPBS (PBS with 0.1% Tween 20), membranes were incubated for 2 h at room temperature with specific Abs. Membranes were then washed twice with TPBS and incubated further at room temperature for 1 h with horseradish peroxidase-conjugated goat antimouse or antirabbit Ab (Jackson ImmunoResearch Laboratories). Membranes were washed twice with TPBS and revealed using an enhanced chemiluminescence detection kit (Amersham).

Measurement of Caspase Activities. Untreated and treated cells were lysed in lysis buffer [10 mM HEPES (pH 7), 40 mM sodium glycerophosphate, 50 mM NaCl, 2 mM MgCl₂, and 5 mM EGTA] and then incubated for 1 h at 37°C in a caspase assay buffer [100 mM HEPES (pH 7.4), 10% glycerol, 0.5

mM EDTA, 0.05% BSA, and 1 mM DTT] containing 20 μ M IETD-AFC or DEVD-AMC (Calbiochem). Caspase activities were measured by monitoring fluorescence continuously in a dual luminescence fluorimeter (LS 50B Perkin-Elmer, Courtabouef, France) using specific excitation and emission wavelengths for IETD-AFC and DEVD-AMC peptide derivative substrates, respectively. Enzyme activities were determined as initial velocities and expressed as relative intensity/min/mg.

RESULTS

The Four Studied Cell Lines Express Only TRAIL-R1 and TRAIL-R2 at Their Cell Surfaces. Flow cytometry analyses performed on nonpermeabilized cells with mAbs raised against the extracellular domains of TRAIL-R1 and TRAIL-R2, respectively, demonstrated that the four tested colon cancer cell lines expressed both receptors at their cell surface (Fig. 1A). The level of expression of the two receptors was similar in each individual cell line. This level of expression differed from one cell line to another, with HCT116 cells expressing the highest level and HT29 cells expressing the lowest level of both proteins. In contrast, the two decoy receptors TRAIL-R3 and TRAIL-R4 were not detected at the cell surface of the four cell lines (for example see results obtained in HT29 cells; Fig. 1B), whereas the tested mAbs could detect these receptors at the cell surface of K562 human leukemic cells (not shown). Because mRNAs encoding these two proteins could be detected in the four cell lines

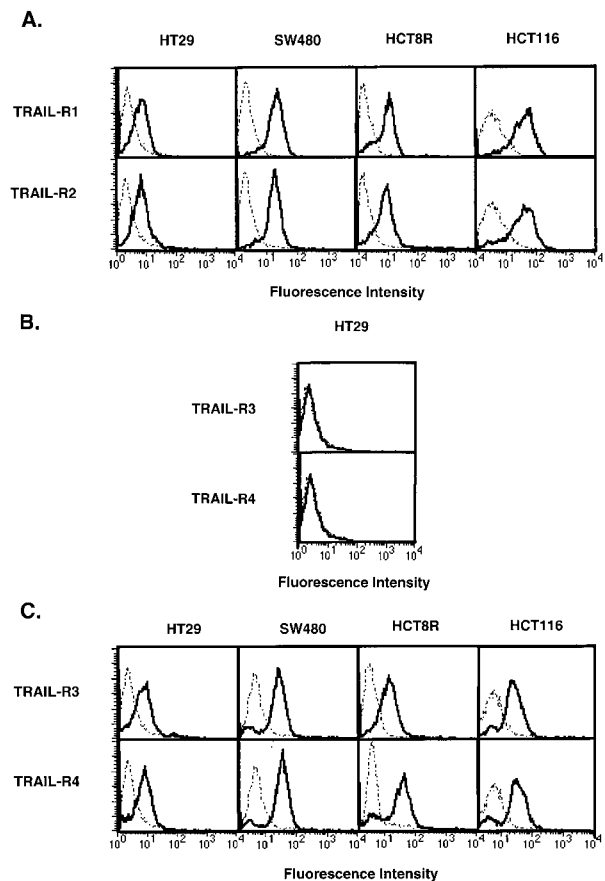


Fig. 1. Expression of TRAIL receptors in four human colon cancer cell lines, as determined by flow cytometry. *A*, membrane expression of TRAIL receptors was studied by using mAbs raised against extracellular domains of TRAIL-R1 (M271) and TRAIL-R2 (M413), respectively. *B*, membrane expression of decoy TRAIL receptors was studied by using mAbs raised against extracellular domains of TRAIL-R3 (M431) and TRAIL-R4 (M445). *C*, intracellular expression of decoy TRAIL receptors was studied by using the same mAbs as in *B* to stain saponin-permeabilized cells. — and ----, the specific and the isotype-matched control mAbs, respectively. One representative of three different experiments is shown.

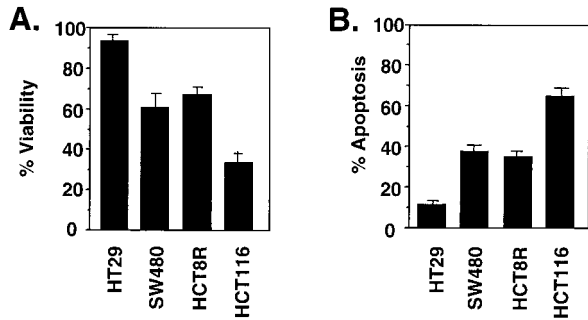


Fig. 2. Tumor cell sensitivity to TRAIL. The four human colon cancer cells were treated for 24 h with recombinant human soluble TRAIL (100 ng/ml + 2 μ g/ml enhancer). A, viability was measured by a colorimetric assay using methylene blue. B, apoptosis was determined by staining cells with Hoechst 33342. Results are the means of three independent experiments; bars, SD.

using reverse transcription-PCR (not shown), we further looked at their expression in the studied cells after permeabilization of the plasma membrane with saponin. As demonstrated on Fig. 1C, TRAIL-R3 and TRAIL-R4 were identified in the four cell lines after permeabilization.

Differential Induction of Apoptosis by Soluble TRAIL in the Four Studied Cell Lines. We used a methylene blue cytotoxic assay to determine the sensitivity of the four colon carcinoma cell lines to the cytotoxic activity of 100 ng/ml TRAIL for 24 h. As demonstrated in Fig. 2A, cell sensitivity to TRAIL correlated with the expression of TRAIL-R1 and TRAIL-R2 on their plasma membrane, with HT29 cells being the most resistant and HCT116 cells being the most sensitive to TRAIL-induced cytotoxicity (Fig. 1A). The cytotoxic activity of TRAIL was found to be related to its ability to trigger apoptotic cell death (Fig. 2B). The specificity of TRAIL-induced apoptosis was demonstrated in HCT116 cells by showing its inhibition in the presence of the neutralizing mAb RIK-2 directed against human TRAIL (Fig. 3). Both TRAIL-R1 and TRAIL-R2 are required for TRAIL-delivered cell death signal because a combination of neutralizing goat Abs directed against the extracellular domain of each receptor is required to completely inhibit TRAIL-induced colon carcinoma apoptosis, whereas each Ab tested alone only partially inhibits cell death induced by the cytokine (Fig. 3).

Cisplatin and Doxorubicin Sensitize Colon Cancer Cells to TRAIL-induced Cytotoxicity. It was shown recently that subtoxic concentrations of cytotoxic drugs could sensitize tumor cells from various origins to TRAIL-mediated cell death (35). We tested the ability of the anthracycline doxorubicin and the alkylating agent cisplatin to increase TRAIL-induced cytotoxicity toward the three colon carcinoma cell lines that had demonstrated some resistance to the cytokine tested alone (see Fig. 2). At concentrations ranging from 0.86 to 8.6 μ M for doxorubicin or from 8 to 16 μ M for cisplatin, the two cytotoxic agents demonstrated a limited cytotoxic effect on the three tested cell lines. As shown on Fig. 4, TRAIL-induced cytotoxicity was significantly enhanced by cotreatment with low concentrations of either doxorubicin or cisplatin. This effect was the most obvious in HT29 cells that were the most resistant cells to TRAIL used alone. Morphological studies indicated that cytotoxic drugs enhanced TRAIL-mediated apoptosis that was inhibited by the neutralizing monoclonal antibody RIK-2 (data not shown) and the caspase inhibitors z-VAD-fmk, z-DEVD-fmk, and z-IETD-fmk (for example, see the results obtained on HT29 cells exposed to doxorubicin in Fig. 5).

Cytotoxic Drugs Do Not Increase the Expression of TRAIL-R1 and TRAIL-R2 on Tumor Cell Plasma Membrane. Etoposide has been shown to increase the expression of TRAIL-R1 and TRAIL-R2

in the cytoplasm of epithelial cell-derived cancer cells (33). We used flow cytometry to determine whether this mechanism could account for the sensitization of colon carcinoma cell lines to TRAIL-mediated apoptosis by low concentrations of cytotoxic drugs. As shown on Fig. 6, neither doxorubicin (0.86 or 8.6 μ M for 24 h) nor cisplatin (8 or 16 μ M for 24 h) significantly increased the expression of TRAIL-R1 and TRAIL-R2 on the plasma membrane of the three studied cell lines. We also observed that these cytotoxic agents had no effect on the expression of TRAIL-R3 or TRAIL-R4 examined in permeabilized cells (data not shown).

Cytotoxic Drugs Sensitize Tumor Cells to TRAIL by Increasing TRAIL-mediated Caspase-8 and Caspase-3 Activation. We further analyzed the molecular mechanisms of TRAIL-induced apoptosis in cells cotreated with nontoxic concentrations of cisplatin or doxorubicin. By using immunoblotting, we observed that cytotoxic drugs increased the ability of TRAIL to induce the cleavage of the M_r 55,000 proform of caspase-8 into its M_r 41,000 and M_r 38,000 active fragments, the cleavage of the caspase-target PARP from a native M_r 116,000 protein into a M_r 85,000 fragment and the cleavage of the BH3 domain only-containing protein Bid, a proapoptotic protein of the Bcl-2 family, from a native M_r 21,000 protein to a p15 fragment (Fig. 7). In accordance with the cleavage of procaspase-8 and PARP, the use of peptide substrates that mimic the target cleavage site of caspase-8 (IETD-AFC) and caspase-3 or caspase-7 (DEVD-AMC) demonstrated that cytotoxic drugs increased the ability of TRAIL to activate these caspases in the two studied colon cancer cell lines (Fig. 8).

DISCUSSION

The present study provides several new insights on the molecular mechanisms of TRAIL-induced colon carcinoma cell apoptosis by showing that: (a) only TRAIL-R1 and TRAIL-R2 are expressed on the plasma membrane of these cells, whereas the decoy receptors cannot be detected at the cell surface; (b) colon cancer cell sensitivity correlates with the level of expression of TRAIL-R1 and TRAIL-R2 death receptors; (c) cell sensitization to TRAIL by nontoxic concentrations of chemotherapeutic drugs is not mediated by an increased expression of these receptors; and (d) anticancer agents sensitize the most resistant cell lines to TRAIL-induced apoptosis by increasing the activation of procaspase-8.

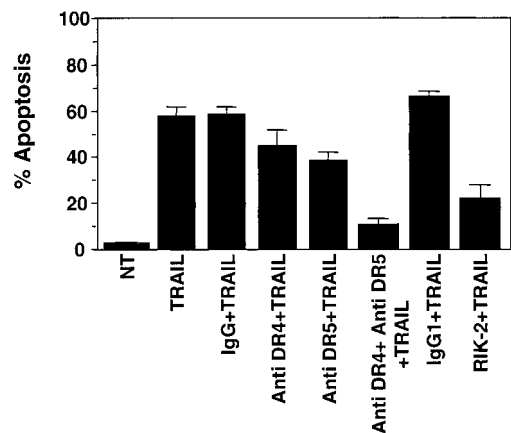


Fig. 3. TRAIL-induced apoptosis is specific for the ligand TRAIL and requires expression of both TRAIL-R1 and TRAIL-R2. Inhibition of TRAIL-induced HCT116 cell apoptosis by the neutralizing anti-TRAIL mAb RIK-2 (10 μ g/ml) or by neutralizing goat IgG anti-TRAIL-R1 (anti-DR4; 1 μ g/ml) and TRAIL-R2 (anti-DR5; 1 μ g/ml). An isotype-matched control IgG1 mAb (10 μ g/ml) or goat IgG (1 μ g/ml) were used as controls. Apoptosis was determined by staining cells with Hoechst 33342. Results are the means of three independent experiments; bars, SD.

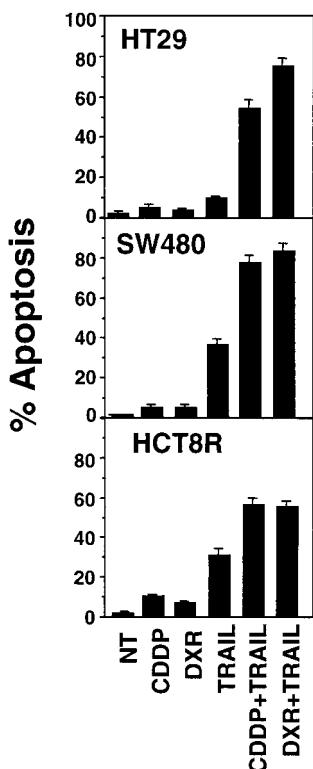


Fig. 4. Cisplatin and doxorubicin sensitize human colon cancer cells to TRAIL-induced apoptosis. Cells were treated for 24 h with either cisplatin (CDDP) alone (HT29 and SW480 cells: 16 μM ; HCT8R: 8 μM); doxorubicin (DXR) alone (HT29 cells: 8.6 μM ; SW480 and HCT8R: 0.86 μM); TRAIL alone (100 ng/ml with 2 $\mu\text{g}/\text{ml}$ enhancer); or combinations of a cytotoxic drug and TRAIL. Apoptosis was determined by staining cells with Hoechst 33342. Results are the means of three independent experiments; bars, SD.

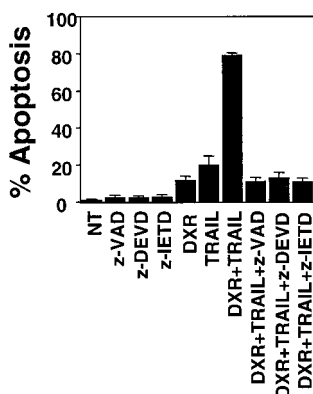


Fig. 5. Doxorubicin (DXR)-induced sensitization to TRAIL depends on caspase activation. HT29 cells were treated for 24 h with 8.6 μM DXR alone, 100 ng/ml TRAIL alone, or a combination of both in the presence or absence of the caspase inhibitors z-VAD-fmk, z-DEVD-fmk, or z-IETD-fmk at the concentration of 100 μM , respectively. Apoptosis was determined by staining cells with Hoechst 33342. Results are the means of three independent experiments; bars, SD.

Identification of TRAIL decoy receptors that bind TRAIL with comparable affinity (<1 nm) to TRAIL-R1 and TRAIL-R2 (14, 15) but do not mediate apoptosis upon ligation because of the lack (TRAIL-R3) or truncation (TRAIL-R4) of their cytoplasmic domains initially suggested that expression of these receptors might be a key determinant of cell sensitivity to TRAIL-induced cell death (3, 10, 11, 14–16). Then, it was observed that several tumor cell lines that were sensitive to TRAIL actually expressed TRAIL-R3 and TRAIL-R4 mRNA when studied by reverse transcription-PCR or RNase protection assay (3, 15, 33). However, mRNA expression does not reflect

cell-surface expression of the proteins (37). In the present study, although we detected the four receptor mRNAs in the four studied cell lines (not shown), only TRAIL-R1 and TRAIL-R2 could be detected at the cell surface of these cell lines. Permeabilization of the plasma membrane was required to detect the two decoy receptors, even in the most resistant HT29 cells. Thus, neither TRAIL-R3 nor TRAIL-R4 might significantly influence colon cancer cell line sensitivity to TRAIL. In contrast, the spontaneous sensitivity of the four studied colon cancer cell lines was observed to correlate with the level of expression of the two death receptors TRAIL-R1 and TRAIL-R2 at the cell surface. Both receptors are required for TRAIL to mediate a maximal cell death signal. A fifth receptor to TRAIL, osteoprotegerin, which exists in a secreted form and competitively inhibits TRAIL binding to TRAIL-R1 and TRAIL-R2 (38), was proposed to modulate the sensitivity of melanoma cell lines to TRAIL-induced cell death (37). Because the mRNA encoding osteoprotegerin could not be detected in HT29 cells (39), this receptor might not account for the resistance of HT29 cells to TRAIL-induced apoptosis.

Several recent reports described the ability of subtoxic concentrations of chemotherapeutic drugs to sensitize tumor cells that are resistant to TRAIL (33–35, 40). The synergistic cytotoxic effect of genotoxic drugs and TRAIL, which was observed also in multidrug-resistant cell lines (35, 40) and was confirmed in our study, was proposed to be mediated by a transcriptional induction of TRAIL-R1 and TRAIL-R2 (30–33) and to be p53 dependent (30, 32). Up-regulation of TRAIL-R1 and TRAIL-R2 has been studied previously either at the mRNA level (30–33) and by immunoblotting (33). This

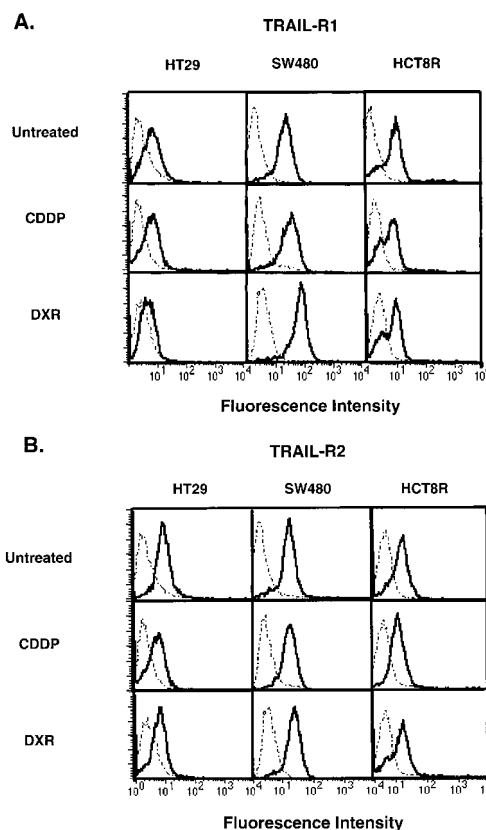
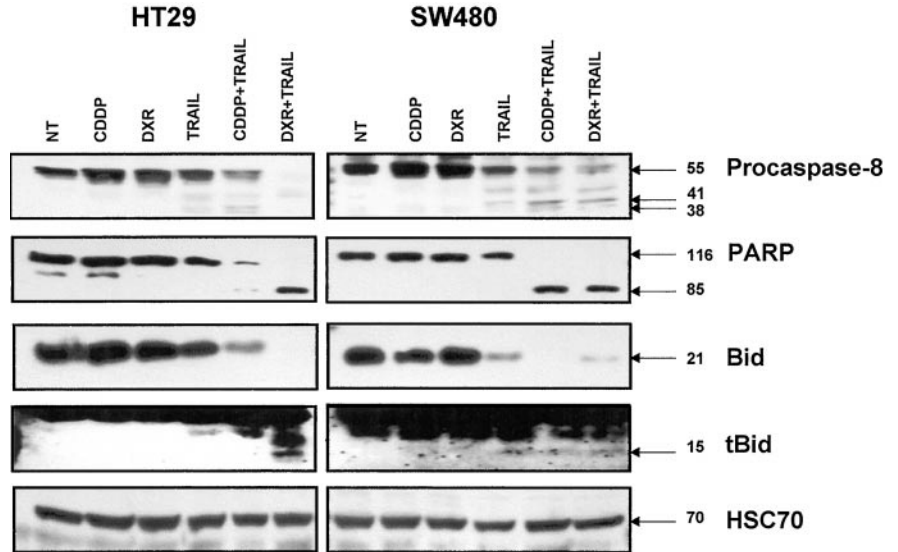


Fig. 6. Cytotoxic drugs do not increase the expression of TRAIL-R1 and TRAIL-R2 on tumor cell plasma membrane. Indicated cells were left untreated or treated for 24 h with cisplatin (CDDP) or doxorubicin (DXR) at doses described in Fig. 4. Then, membrane expression of TRAIL-R1 and TRAIL-R2 was studied by flow cytometry after staining the cells with M271 (A) and M413 (B) mAb, respectively. — and ----, the specific and the isotype-matched control mAbs, respectively. One representative of three different experiments is shown.

Fig. 7. Influence of the cytotoxic drug/TRAIL combination on the expression of procaspase-8, PARP, and Bid. Tumor cells were exposed to TRAIL in the absence or presence of cisplatin (CDDP) or doxorubicin (DXR). HT29 and SW480 were treated for 24 h with the drug alone, recombinant TRAIL alone, or a combination of both at doses described in Fig. 4. The expression of procaspase-8, PARP, and Bid was monitored by Western blot analysis. An antihuman HSC70 antibody was used as a loading control. Numbers are molecular weights in thousands. One representative of three different experiments is shown.



drug-induced up-regulation was not confirmed in the present study in which the expression of both receptors was analyzed at the plasma membrane by flow cytometry. This lack of modulation of TRAIL receptor membrane expression upon exposure to DNA-damaging agents did not depend on p53 status because it was observed in both p53 wild-type and p53-mutated cell lines.

The concentration of cytotoxic drugs used to sensitize tumor cells to TRAIL-induced cell death could account for the discrepancies we observed with other studies. High concentrations of etoposide that are not clinically relevant were shown to up-regulate TRAIL-R1 and TRAIL-R2 mRNA and protein levels in breast cancer cell lines (33), whereas lower, more clinically relevant concentrations of doxorubicin had no effect on the expression of the studied receptors in these cell lines (34). Thus, we cannot rule out that higher concentrations of doxorubicin or cisplatin could have enhanced the expression of TRAIL receptors at the surface of the tested colon carcinoma cells. Discrepancies between studies could also depend on the choice of the cytotoxic drug because low concentrations of doxorubicin were shown to sensitize breast cancer cells to TRAIL, whereas high con-

centrations of 5-fluorouracil were required for obtaining this effect (34).

We have shown previously that the cytotoxic drugs doxorubicin and cisplatin were able to increase the expression of another death receptor, Fas (CD95/APO-1), at the surface of HT29 cells (36). This up-regulation was shown to account for drug-induced sensitization of colon cancer cells to Fas-mediated cell death. The present study indicates that the molecular mechanisms of drug-induced sensitization to TRAIL might be distinct from those of drug-mediated sensitization to Fas agonists. In addition, although a role for Fas in drug-induced cytotoxicity has been established (41), the role of TRAIL receptors in chemotherapeutic drug-mediated cytotoxicity remains to be investigated.

The differential expression of intracellular inhibitors of apoptosis is another mechanism that could account for the differential sensitivity of colon carcinoma cell lines to TRAIL-mediated apoptosis. One of the molecules that can interfere with TRAIL-mediated cell death is c-FLIP (9, 29, 42), a protein structurally related to procaspase-8 but lacking a catalytic active site and the residues that form the substrate binding pocket (28). This protein, which was implicated in the resistance of resting T cells to Fas-induced cell death (43) and in the progression and immune escape of tumors *in vivo* (44, 45), is predominantly found as a M_r 55,000 isoform in most tissues and cell lines (46), although a minor species of M_r 27,000/28,000 was also detected in some cell types (28, 47). The level of c-FLIP expression decreases upon exposure to actinomycin D, which was proposed to account for cell sensitization to TRAIL-induced apoptosis by this inhibitor of transcription (37). In colon cancer cell lines, we did not identify any correlation between c-FLIP expression, as determined by immunoblotting, and spontaneous cell sensitivity to TRAIL nor cell sensitization to TRAIL-mediated cell death by the chemotherapeutic agents doxorubicin and cisplatin (not shown).

Interestingly, cytotoxic drugs lowered the signaling threshold required for TRAIL-induced cell death. Both doxorubicin and cisplatin sensitized colon cancer cells to TRAIL-induced cleavage of procaspase-8 and PARP and caspase activation. These results indicated that anticancer drugs increased the ability of TRAIL to trigger a caspase-dependent cell death, in accordance with observations made in breast cancer cells and described previously (34). Fas-mediated cell death can involve the cleavage of the COOH-terminal part of a BH3 domain-containing proapoptotic member of the Bcl-2 family desig-

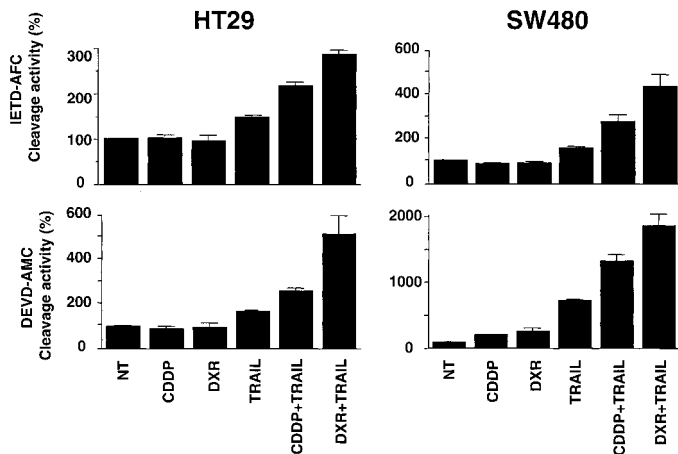


Fig. 8. Influence of the cytotoxic drug/TRAIL combination on caspase-8 and caspase-3 activities. HT29 and SW480 were treated for 24 h with the drug alone, recombinant TRAIL alone, or a combination of both at doses described in Fig. 4. Caspase-8- and caspase-3-like activities were measured in cell extracts by hydrolysis of the IETD-AFC and DEVD-AMC substrates, respectively. Arbitrary units of protease activity were calculated as described in "Materials and Methods." Results are expressed as means of three independent experiments; bars, SD.

nated Bid. Then, translocation of the truncated Bid to mitochondria causes various changes, leading to the activation of effector caspases (48, 49). In accordance with a recent report (27), we show here that similar events are involved in TRAIL-mediated apoptosis. Although they do not seem to modulate the expression of Bid in colon cancer cells, the cytotoxic drugs doxorubicin and cisplatin sensitize these cells to TRAIL-mediated decreased expression of the native form of Bid in its p15 fragment and the activation of effector caspases that cleave PARP (26).

The safety of TRAIL administered *in vivo* to athymic mice either locoregionally (50) or *i.v.* (5, 6) and its efficacy in suppressing tumor growth suggested that TRAIL-based tumor therapy may be an efficient anticancer strategy. The ability of subtoxic concentrations of chemotherapeutic drugs to restore TRAIL-mediated pathway to death in cell lines that are resistant to TRAIL-induced cytotoxicity (34, 35) suggested that combination of an anticancer drug with TRAIL may enforce the TRAIL-based therapeutic strategy. The present study shows that clinically relevant concentrations of genotoxic agents lower the signaling threshold required for TRAIL-induced cell death without increasing the expression of TRAIL receptors. Interestingly, the cytotoxic drugs tested in this study have limited efficacy toward colon cancer cells by themselves, whereas their combination with TRAIL is an effective inducer of tumor cell death *in vitro*. It remains to be determined whether this combination increases the selectivity of anticancer therapy toward tumor cells *in vivo*, as suggested by initial studies testing the combination of TRAIL to 5-fluorouracil (5). Confirmation of the clinical interest of this strategy would be of great importance in colon cancer cells that remain highly resistant to most chemotherapeutic drugs used alone.

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