# Resistance to TRAIL is associated with defects in ceramide signaling that can be overcome by exogenous $C_6$-ceramide without requiring down-regulation of cellular FLICE inhibitory protein

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## Abstract

**Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a member of the tumor necrosis factor superfamily that selectively induces apoptosis in malignant cells. However, not all cancer cells are susceptible to TRAIL and mechanisms of resistance and new strategies to enhance sensitivity are an area of intense investigation. Glucose withdrawal or paclitaxel increase intracellular ceramide, down-regulate cellular FLICE inhibitory protein (cFLIP), and sensitize cells to TRAIL. Therefore, we investigated whether TRAIL resistance is due to ceramide levels and/or defects in ceramide generation following ligand binding. Colon cancer cells isolated from the primary tumor (SW480) and a subsequent metastasis (SW620) of the same patient have different sensitivities to TRAIL. Mass spectrometry was used to compare ceramide content in untreated and TRAIL-treated cells. Overall levels of ceramide were comparable in the cell lines but TRAIL-sensitive SW480 cells contained a higher percentage of $C_{16}$-, and $C_{18}$-ceramide and lower $C_{24}$-ceramides than TRAIL-resistant SW620 cells. Upon TRAIL treatment, ceramide (primarily $C_{16}$-ceramide) increased in SW480 but not SW620 cells. The increase in ceramide occurred with slow kinetics, paralleling caspase-3/7 activation. Combination of $C_6$-ceramide with TRAIL resulted in apoptosis of SW620 cells. However, exogenous $C_6$-ceramide did not affect levels of cFLIP nor did pretreatment sensitize cells to TRAIL. Exposure to TRAIL prior to ceramide was required to induce apoptosis, suggesting that ceramide plays a role in enhancing or amplifying TRAIL-mediated signaling. Our results suggest that ceramide plays a role in promoting TRAIL-mediated apoptosis and that TRAIL-resistant cancers may benefit from combination therapy with ceramide or agents that enhance ceramide accumulation. [Mol Cancer Ther 2005;4(9):1320–7]**

## Introduction

Colorectal cancer is a leading cause of cancer deaths with an estimated 144,000 new cases expected in 2005. Current treatments are often only marginally effective due to the development of drug resistance and toxic side effects. Due to the limited efficacy of these treatments, development of novel therapies could significantly enhance the outcome for colon cancer patients. One potential new anticancer agent is tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL), a death receptor ligand of the TNF superfamily (1, 2). In contrast to other members of the TNF family and for reasons not fully understood, TRAIL seems to selectively kill cancer cells without toxicity towards normal cells (3, 4). Genentech Inc. (San Francisco, CA) has recently gained approval to test a recombinant form of the protein in a phase I clinical trial.

TRAIL induces apoptosis by binding to agonistic TRAIL receptors (DR4 and/or DR5) followed by activation of initiator caspases and subsequent (mitochondria-dependent or -independent) activation of effector caspases that are responsible for execution of apoptosis (5). Numerous proteins such as cellular FLICE inhibitory protein (cFLIP), antiapoptotic members of the Bcl-2 family, and inhibitors of apoptosis negatively regulate this process. Because not all cancer cells are sensitive to TRAIL, mechanisms of resistance and new strategies to enhance tumor cell–killing are an area of intense investigation. Recent studies have shown that glucose withdrawal or paclitaxel increase intracellular ceramide levels and sensitize prostate and renal cancer cells to TRAIL (6, 7).

The sphingolipid ceramide plays a role in various cellular responses including apoptosis. Ceramide levels increase in response to stress stimuli such as TNF, FasL, chemotherapeutic agents, and radiation (8). In colon tumors, ceramide levels are significantly lower than in the normal mucosa (9). The same study found that increasing ceramide levels by ceramidase inhibitors resulted in an apoptotic response and prevention of liver

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metastases in a mouse model. The role of ceramide during apoptosis is not completely understood, but it is in many cases required during the initiation phase and could also play a role as an amplification signal. Biological targets of ceramide include ceramide-activated protein phosphatases, which dephosphorylate c-*Jun*, Rb, PKC, Akt, SR, and Bcl-2 proteins (8). Akt positively regulates the expression of cFLIP, an antiapoptotic protein that interferes with caspase-8 processing and activation by heterodimer formation (10). High levels of Akt activity and/or cFLIP expression have been associated with resistance to TRAIL (7, 11–13). Generation of ceramide inhibits Akt activation and down-regulates cFLIP expression (6, 7).

In this study, we investigated whether TRAIL resistance may be due to differences in ceramide levels and/or a defect in ceramide generation following ligand binding. Overall levels of ceramide were comparable in two cell lines derived from the same patient but differences were detected in the distribution of ceramide species. Following TRAIL exposure, ceramide increased in TRAIL-sensitive but not TRAIL-resistant cells. TRAIL resistance could be reversed by exogenous ceramide. However, in contrast to ceramide generated following paclitaxel or glucose withdrawal, exogenous ceramide did not sensitize cells to TRAIL via cFLIP down-regulation but seemed to promote apoptosis after ligand binding.

## Materials and Methods

### Cell Lines and Culture

The cell lines SW480 and SW620 were purchased from the American Type Culture Collection (Rockville, MD). Cell lines were cultured in Primaria plasticware (Falcon, Bedford, MA) and maintained in RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% fetal bovine serum (Hyclone, Logan, UT). Cell cultures were maintained at 37°C in a 5% $CO_2$ atmosphere.

### Viability and Caspase-3/7 Assays

For the CellTiter Aqueous One Solution Cell Proliferation (MTS) assay and the Apo-ONE homogeneous caspase-3/7 assay (both from Promega, Madison, WI), cells were seeded into 96-well plates at $2 \times 10^4$ cells per well, incubated overnight and subsequently treated with Killer-TRAIL (Alexis, Lausanne, Switzerland) and/or D-*erythro*-$C_6$-ceramide and its inactive enantiomer L-*erythro*-$C_6$-ceramide which were kindly provided by the Medical University of South Carolina Lipodomics Core facility. The substrate for the caspase assay was added after 1 to 24 hours of treatment (as indicated in the text) and plates read 2 to 3 hours later using a FluoStar plate reader (BMG Labtechnologies Inc., Durham, NC) with emission and excitation wavelengths of 485 and 520 nm, respectively. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent for the CellTiter Cell Proliferation assay was added 24 hours after initiation of treatment and plates read at an absorbance of 490 nm 1 to 2 hours later using a $V_{max}$ kinetic microplate reader (Molecular Devices, Sunnyvale, CA). All treatments were done in triplicate. Background fluorescence or absorbance was determined by incubating media with substrate alone and subtracting the values from wells containing cells.

### Flow Cytometry

Cells were seeded into six-well plates at $6 \times 10^5$ cells per well, incubated overnight and subsequently treated with Killer-TRAIL and/or $C_6$-ceramide. After overnight incubation (16–20 hours), nonadherent and adherent cells were combined, washed in PBS and either incubated with Via-Probe (BD PharMingen, San Diego, CA) or fixed in 70% ethanol overnight at 4°C for propidium iodide staining. Cells were analyzed on a FACSCalibur (Becton Dickinson, Bedford, MA) in the Medical University of South Carolina Flow Cytometry Core Facility. A mini-mum of 10,000 events was scored for each sample.

### Ceramide Determination

Cells were plated overnight and, if indicated, treated with 100 ng/mL TRAIL prior to harvesting. At indicated time points, cells were scraped into the media, pelleted at 2,500 rpm for 5 minutes, washed with PBS and repelleted. Cell pellets were frozen at −80°C and provided to the Lipodomics Core Facility at Medical University of South Carolina for ceramide analysis.

AESI/MS/MS analysis of endogenous ceramide species were done on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer, operating in a multiple reaction monitoring–positive ionization mode, using a modified version of the published protocol (14). Cell pellets, corresponding to about 1 to $2 \times 10^6$ cells, were fortified with the internal standards [$C_{17}$ base D-*erythro*-sphingosine (17CSph), $C_{17}$ sphingosine-1-phosphate (17CSph-1P), *N*-palmitoyl-D-*erythro*-$C_{13}$ sphingosine (13C16-Cer) and heptadecanoyl-D-*erythro*-sphingosine (C17-Cer)], and extracted with ethyl acetate/isopropanol/water (60:30:10 v/v) solvent system. After evaporation and reconstitution in 100 μL of methanol, samples were injected on the Surveyor/TSQ 7000 LC/MS system and gradient eluted from the BDS Hypersil C8, 150 × 3.2 mm, 3 μm particle size column, with 1.0 mmol/L methanolic ammonium formate / 2 mmol/L aqueous ammonium formate mobile phase system. Peaks corresponding to the target analytes and internal standards were collected and processed using the Xcalibur software system.

Quantitative analysis was based on the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the internal standards. The target analyte/internal standard peak areas ratios were plotted against analyte concentration. The target analyte/internal standard peak area ratios from the samples were similarly normalized to their respective internal standards and compared with the calibration curves, using a linear regression model.

### Antibodies and Western Blot Analysis

Cells were seeded at $1.4 \times 10^6$/60 mm plate incubated overnight and subsequently treated with $C_6$-ceramide (30 μmol/L) for the indicated times. Cells were rinsed

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with PBS and lysed in radioimmunoprecipitation assay buffer (1% NP40, 0.5% deoxycholate, 0.1% SDS in PBS) containing a mammalian protease inhibitor cocktail (P-8340 Sigma, St. Louis, MO). Lysates were centrifuged (20,000 × *g*) prior to performing protein assays on the supernatant (detergent-compatible protein assay, Bio-Rad, Hercules, MA). Protein (50 μg) was separated on 4% to 12% Bis/Tris NuPage gels in MES buffer (Novex/Invitrogen) and transferred to nitrocellulose (Bio-Rad). After blocking in 5% milk in TBS-Tween for 1 hour, membranes were incubated with anti-FLIP antibody (NF-6, 1:3) in TBS-Tween overnight at room temperature. After washing, membranes were incubated with horseradish peroxidase–conjugated anti-mouse IgG (1:5,000, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and the chemiluminescent signal detected with DuraWest Supersignal (Pierce Biotechnology Inc., Rockford, IL). To ensure equal loading, membranes were reprobed with 1:2,000 anti-actin (Sigma) followed by 1:100,000 horseradish peroxidase–conjugated anti-rabbit IgG (Santa Cruz Biotechnology).

## Results

### Differential Susceptibility of Colon Cancer Cell Lines to TRAIL

The goal of this study was to determine whether differences in ceramide levels or aberrant ceramide generation following ligand binding play a role in resistance to TRAIL. Two colon cancer cell lines, derived from the same patient, were used for this purpose. SW480 cells were established from the primary adenocarcinoma of the colon, whereas SW620 cells were derived from a metastasis taken 1 year later. As shown in Fig. 1A, exposure of SW480 cells to increasing concentrations of recombinant TRAIL resulted in a dose-dependent decrease in viability. In contrast, viability of SW620 cells remained unaffected. To confirm that the response to TRAIL was apoptotic, we did caspase-3/7 activity assays (Fig. 1B). In SW480 cells, a 4- or 6- to 7-fold increase in caspase activity was measured at 5 or 24 hours, respectively, following exposure to TRAIL. In contrast, a <2-fold increase in caspase activity was detected in SW620 cells. This level of caspase activation may be insufficient to cause cell death. TRAIL resistance in SW620 cells was not due to differences in expression of various proteins of the apoptotic pathway, including TRAIL receptors, caspase-8, cFLIP, Bax, Bcl-2, caspase-3, or X-linked mammalian inhibitor of apoptosis protein (data not shown). Although the TRAIL phenotype of SW480 and SW620 cells has not, to our knowledge, been compared directly, other investigators using different TRAIL preparations and methodologies have also described SW480 as sensitive and SW620 as resistant (3, 15–17).

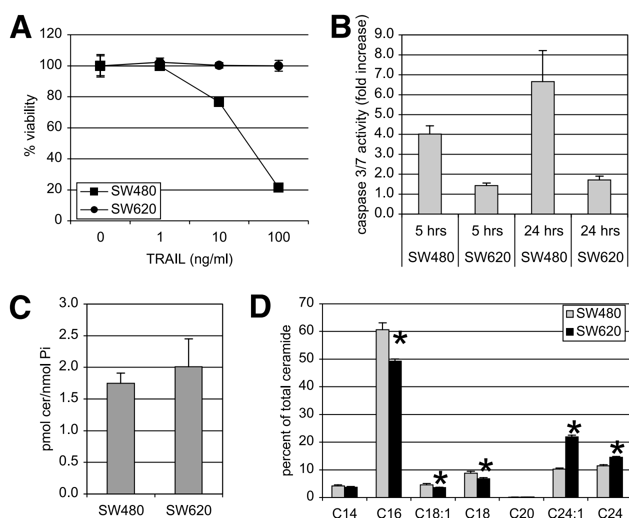


**Figure 1.** Comparison of TRAIL sensitivity and ceramide content in SW480 and SW620 cells. **A,** viability was measured 24 h after exposure to increasing concentrations of TRAIL. **B,** caspase-3/7 activity was determined after incubation with 100 ng/mL TRAIL. Similar results have been obtained in at least three independent experiments. **C,** total ceramide content in SW480 and SW620 cells are similar ($P = 0.19$). **D,** the amount of ceramide species expressed as a percentage of total ceramide from $1 \times 10^6$ cells; *, $P < 0.001$; *columns*, mean; *bars*, ± SD from three experiments.

### SW480 and SW620 Cells Have Similar Levels of Ceramide with Differential Distribution of Ceramide Species

Previously, it has been shown that tumor samples from colon cancer patients contain at least 50% less ceramide than the normal mucosa (9). In an animal model, treatment with ceramidase inhibitors, which allow ceramide to accumulate by preventing its metabolism, resulted in apoptosis and prevented establishment of colon cancer liver metastases, suggesting that lower levels of ceramide might be associated with an impaired apoptotic response (9). Thus, we considered the possibility that SW620 cells have lesions in ceramide metabolism that lead to TRAIL resistance. Initially, we compared whether overall levels of ceramide are lower in TRAIL-resistant SW620 cells compared with TRAIL-sensitive SW480 cells. Lipids from each cell line were extracted and analyzed for ceramide content by mass spectrometry. Both cell lines contained similar levels of total ceramide (Fig. 1C). Because mass spectrometry allows analysis of individual ceramide species, we also compared the distribution of ceramides. The most abundant species of ceramide in each cell line was $C_{16}$-ceramide, followed by $C_{24:0}$ and $C_{24:1}$ ceramides. $C_{14}$, $C_{18}$ and $C_{18:1}$ ceramides each represented <10% of the total ceramide content and $C_{20}$ ceramide was the least abundant (Fig. 1D) TRAIL-sensitive SW480 cells had significantly higher levels of long chain ceramides, particularly $C_{16}$-ceramide, whereas TRAIL-resistant SW620 cells contained more of the very long ceramides ($C_{24:0}$ and $C_{24:1}$). These differences were statistically significant ($P < 0.001$), and may be of functional significance (see Discussion).

### $C_{16}$-Ceramide Increases following TRAIL Treatment in SW480 but not SW620 Cells

Next, we determined whether ceramide levels changed after TRAIL treatment. Following exposure to TRAIL for 4 hours, $C_{16}$-ceramide increased significantly in SW480 but not SW620 cells (Fig. 2A). Other long-chain ceramides ($C_{14}$, $C_{18:0}$, $C_{18:1}$) also increased by at least 50% in SW480 cells

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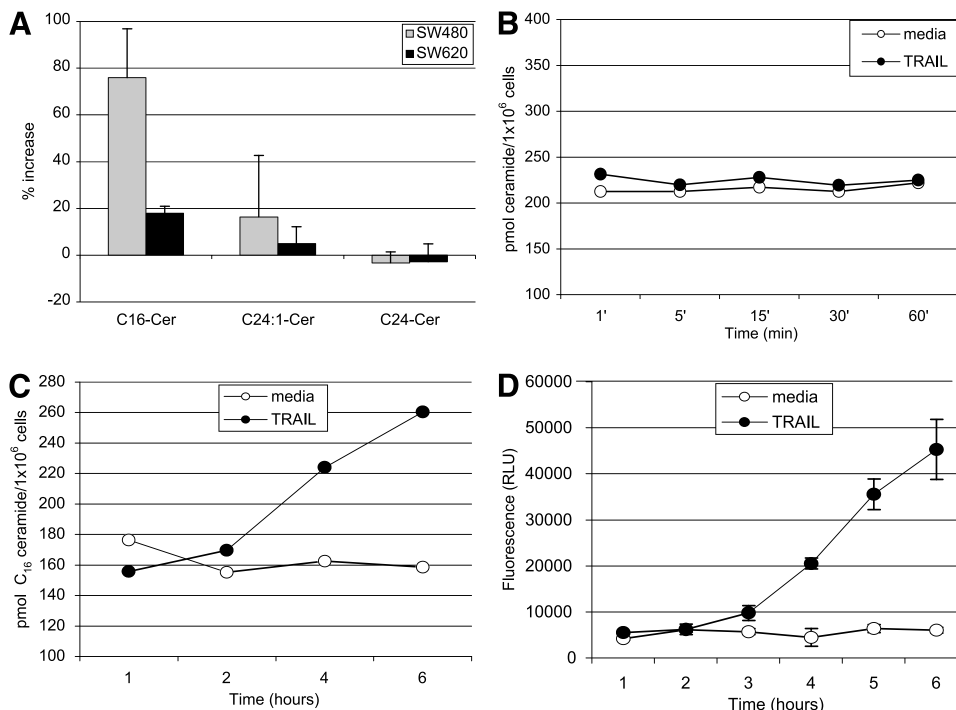


**Figure 2.** Ceramide generation and caspase-3/7 activation following TRAIL treatment. **A,** cells were treated with 100 ng/mL TRAIL for 4 h and ceramide levels were compared with untreated cells. *Columns*, mean; *bars*, ± SD from four experiments. SW480 cells were treated with 100 ng/mL TRAIL for the indicated times followed by analysis of ceramide (**B** and **C**) or caspase-3/7 activity (**D**). A representative experiment is shown.

(data not shown), whereas very long chain ceramides ($C_{24:0}$, $C_{24:1}$) were not significantly elevated following TRAIL treatment in either cell line (Fig. 2A). Longer incubation with TRAIL did not lead to an increase of $C_{16}$-ceramide (or other ceramide species) in SW620 cells (data not shown).

In some experimental systems, for example, ligation of Fas by an anti-CD95 antibody in U937 cells or treatment of HT29 colon carcinoma cells with cisplatin, ceramide increases rapidly (within minutes), and then declines to baseline levels (18, 19). In other studies, ceramide accumulation occurs with slower kinetics and continues to increase with time (20, 21). Because the kinetics of ceramide generation may depend on the stimulus and/or cell line, we did short and long time course experiments. We did not observe any changes in ceramide levels within the first hour of TRAIL treatment (Fig. 2B). Ceramide levels began to increase after 2 hours and continued to increase linearly up to 6 hours, which was the latest time point examined in our experiments. The elevation in ceramide levels was primarily due to $C_{16}$-ceramide, which represents about 60% of the total ceramide in these cells (Fig. 2C). Other long-chain ceramides displayed a similar pattern, but $C_{24:0}$ ceramide did not increase (data not shown). The increase in $C_{16}$-ceramide closely paralleled caspase-3/7 activation (Fig. 2D).

### TRAIL Resistance Is Reversed by Exogenous $C_6$-Ceramide

Because $C_{16}$-ceramide levels increased in TRAIL-sensitive but not TRAIL-resistant cells, it was of interest to determine whether exogenous ceramide could sensitize SW620 cells to TRAIL. For this purpose, we used the cell-permeable ceramide analogue D-e-$C_6$-ceramide, which is metabolized primarily to $C_{16}$-ceramide and should therefore be relevant to this study (22). In a viability assay, that measures mitochondrial activity, the $IC_{50}$ of $C_6$-ceramide in SW620 cells was 45 μmol/L (data not shown). Exposure of SW620 cells to 30 μmol/L $C_6$-ceramide for 24 hours resulted in rounding but not cell death (Fig. 3B). The morphology of SW620 cells in the presence of TRAIL resembled that of untreated cells, although occasional apoptotic cells were observed (Fig. 3C). This is consistent with the low level of caspase activity following TRAIL treatment (Fig. 1B). However, combination of $C_6$-ceramide and TRAIL caused

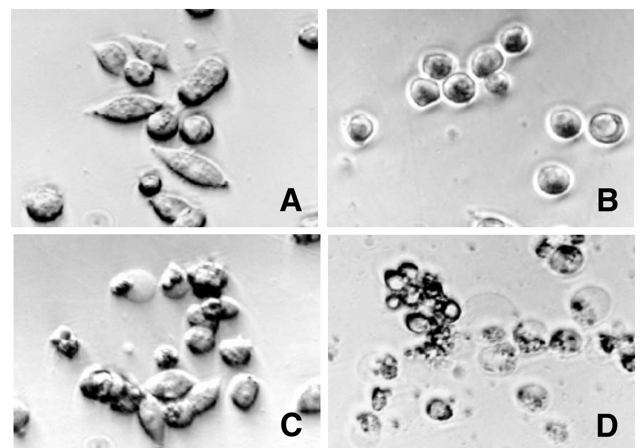


**Figure 3.** Morphologic assessment of SW620 cells following treatment. Cells were untreated (**A**), treated with 30 μmol/L ceramide (**B**), 100 ng/mL TRAIL (**C**), or both (**D**). Images were captured using a Zeiss Axiovert 200 at a magnification of ×200.

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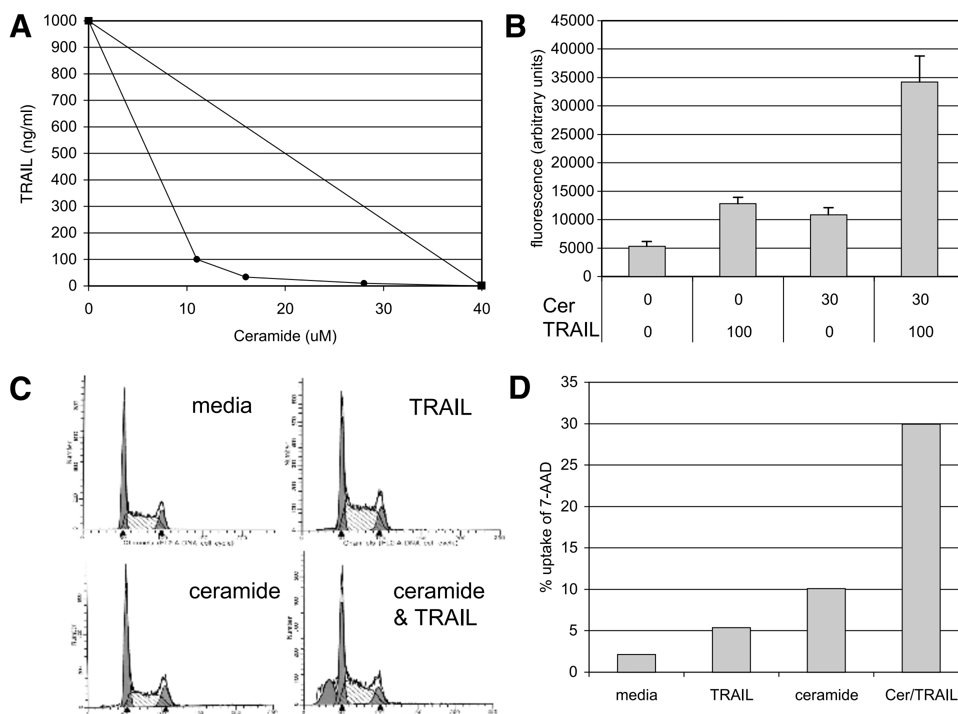


**Figure 4.** Combination of TRAIL and ceramide in SW620 cells. **A,** synergistic effect of TRAIL and C6-ceramide in the viability assay. Caspase-3/7 activity (**B**), propidium iodide staining (**C**), and membrane permeability (**D**) following incubation with 100 ng/mL TRAIL, 30 μmol/L ceramide, or both.

virtually all cells to lose adherence and undergo cell death (Fig. 3D). The response following combination of TRAIL and ceramide was synergistic (Fig. 4A). We also evaluated the effect of ceramide and TRAIL on caspase-3/7 activity (Fig. 4B), generation of an apoptotic sub-$G_1$ peak by propidium iodide staining (Fig. 4C), and membrane permeability using Via-Probe (Fig. 4D). In each assay, the effect of ceramide and TRAIL was greater than additive. Generation of a sub-$G_1$ peak and increased caspase activity indicate that cells treated with ceramide and TRAIL were undergoing apoptosis. L-e-$C_6$-ceramide (the inactive enantiomer of D-e-$C_6$-ceramide) did not affect viability of SW620 cells either alone or in combination with TRAIL (Fig. 5A). Interestingly, $C_6$-ceramide did not further enhance TRAIL sensitivity in SW480 cells (Fig. 5B).

#### Treatment with Ceramide and TRAIL Does Not Result in Subsequent Resistance

To determine the long-term effect of TRAIL and ceramide treatment, cells were exposed to TRAIL, ceramide or ceramide and TRAIL for 24 hours, and then cultured in growth medium for an additional 3 days. On day 4, cells were counted using a hemacytometer. There was no difference in cell number between untreated and TRAIL-treated cells (Fig. 5C). Ceramide treatment resulted in a significantly lower amount of cells. This is likely attributable to growth inhibition, because $C_6$-ceramide could induce $G_0$/$G_1$ cell cycle arrest (23). There were too few cells to count in the wells that received ceramide/TRAIL combination treatment although an occasional adherent cell was observed. We allowed SW620 cells treated with ceramide or ceramide and TRAIL to expand and established the sublines SW620C (ceramide-treated) and SW620CT (ceramide- and TRAIL-treated). To investigate the possibility that we had selected for a resistant subset of cells following treatment, the response of SW620C and SW620CT to TRAIL, ceramide or a combination of both were compared with parental SW620 cells. Like the parental cells, SW620C and SW620CT underwent cell death following exposure to both TRAIL and ceramide (Fig. 5D).

#### Exogenous $C_6$-Ceramide Does Not Reduce Levels of cFLIP

One current hypothesis regarding the role of ceramide during TRAIL-induced apoptosis is that ceramide inhibits the expression of cFLIP via the Akt-nuclear factor κB axis. cFLIP is an antiapoptotic protein that forms heterodimers with procaspase-8, preventing homodimer formation and generation of the active caspase-8 tetramer. Selective down-regulation of cFLIP by antisense oligomers or small interfering RNA sensitizes cells to death receptor ligands (24, 25). Surprisingly, exogenous $C_6$-ceramide did not affect levels of either the long or short isoform of cFLIP (Fig. 6A), suggesting that ceramide enhances TRAIL sensitivity by a cFLIP-independent mechanism.

#### Effects of Sequential Ceramide and TRAIL Treatment

To further investigate the role of $C_6$-ceramide during TRAIL-induced apoptosis, we determined whether the sequence of exposure is important in eliciting an apoptotic response. SW620 cells were treated with either $C_6$-ceramide or TRAIL for 3 or 5 hours, followed by the reciprocal treatment (or media as control) for an additional 20 hours. As shown in Fig. 6B, sequential treatment with ceramide

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followed by TRAIL was ineffective. These results are in agreement with our observation that ceramide does not reduce levels of cFLIP. However, when TRAIL was added first, followed by ceramide, significant cell death was observed, suggesting that ceramide may play a role in promoting TRAIL-mediated apoptosis.

## Discussion

In this study, we tested the hypothesis that resistance to TRAIL may be due to defects in ceramide metabolism. This hypothesis was based on previous studies that show (*a*) ceramide levels are decreased in colon tumors compared with the normal mucosa, (*b*) restoration of ceramide levels by ceramidase inhibitors induces apoptosis and prevents the establishment of liver metastases in an animal model, and (*c*) low glucose or paclitaxel sensitize cells to TRAIL which correlated with an increase in ceramide levels.

One mechanism by which resistance could develop is aberrant ceramide signaling. Although we did not find significant differences in the overall ceramide levels between SW480 and SW620 cells, statistical differences were detected upon comparison of individual ceramide species. As a percentage of total ceramide content, SW480 cells contained more $C_{16}$-and $C_{18}$- and less $C_{24}$-ceramides than SW620 cells. Specific gene products have recently been shown to synthesize ceramide with a high degree of fatty acid specificity. These gene products constitute a new subfamily of translocating chain–associating membrane protein homologues and include TRH-1 and TRH-4 (26, 27). Overexpression of TRH-1 corresponded with increased generation of $C_{18}$- and $C_{20}$-ceramides, whereas TRH-4 overexpression resulted in a strong increase in $C_{16}$-ceramide (26). In a majority of head and neck cancers, a selective decrease in $C_{18:0}$-ceramide was observed that could be restored to normal levels by overexpression of murine TRH-1 (mLAG1). Restoration of $C_{18:0}$-ceramide resulted in 70% to 80% growth inhibition by modulating telomerase activity and inducing apoptosis (28). These findings suggest that differences in ceramide species in SW480 and SW620 cells may be of functional significance that warrants further investigation.

SW480 cells had a higher proportion of $C_{16}$-ceramide, which further increased upon TRAIL treatment. Other studies have also observed a specific increase in $C_{16}$-ceramide during apoptosis. For example, apoptosis induced by FasL or radiation resulted in a specific increase in mitochondrial $C_{16}$-ceramide (29) and Thomas et al. found that an increase in $C_{16}$-ceramide closely paralleled the decrease in mitochondrial mass (30). Apoptosis induced by cross-linking the B cell receptor also results in increased $C_{16}$-ceramide levels during early apoptosis (21). Despite being the major ceramide species, there was proportionally less $C_{16}$-ceramide in SW620 cells, and levels did not increase significantly following TRAIL treatment. Levels of $C_{24}$-ceramide were not affected by TRAIL in either cell line. Similarly, Kroesen et al. (21) did not detect an increase in $C_{24}$-ceramides during early apoptosis. $C_{24}$-ceramide has been shown to increase as a function neutral sphingomyelinase 2 correlating with growth arrest (23).

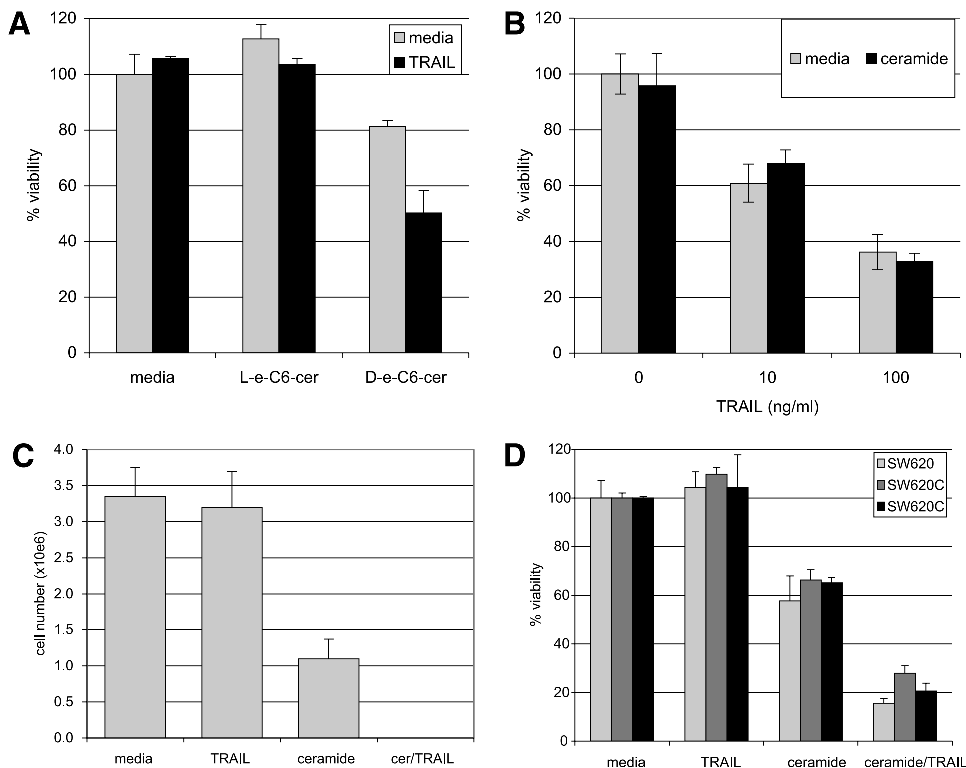


**Figure 5.** Specificity and long-term effects of $C_6$-ceramide during TRAIL-induced apoptosis. **A,** SW620 cells were incubated with 20 μmol/L D-e-$C_6$-ceramide or L-e-$C_6$-ceramide in the presence and absence of 100 ng/mL TRAIL. **B,** SW480 cells were incubated with TRAIL in the presence and absence of 30 μmol/L $C_6$-ceramide. **C,** SW620 cells were incubated with 100 ng/mL TRAIL, 30 μmol/L $C_6$-ceramide, or both for 24 h followed by incubation in growth medium for 3 d. **D,** sensitivity to 100 ng/mL TRAIL, 30 μmol/L ceramide, or both was determined in parental SW620 cells as well as sublines established from ceramide (*SW620C*) and ceramide/TRAIL (*SW620CT*) sublines. Data are from a representative experiment. Similar results have been obtained in other experiments.

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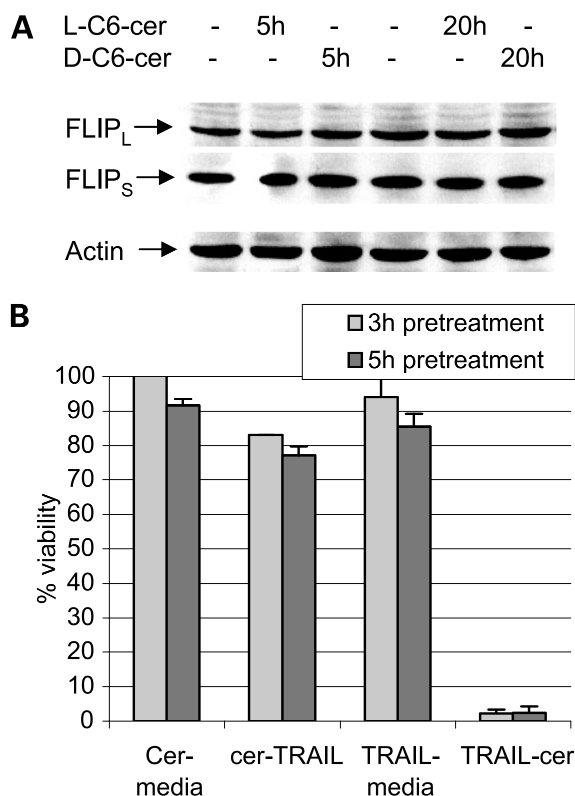


**Figure 6.** Effects of $C_6$-ceramide on FLIP and sequential treatment. **A,** Western blot analysis of cFLIP isoforms in SW620 cells following incubation without or with 30 μmol/L ceramide. Actin is included as a loading control. **B,** viability of SW620 cells incubated sequentially with 30 μmol/L ceramide and 100 ng/mL TRAIL. Data are from a representative experiment. Similar results have been obtained in at least three independent experiments.

Thus, it seems that increases in specific ceramide species may be associated with specific cellular responses. The higher levels of $C_{16}$-ceramide thus suggest a more ''proapoptotic'' ceramide phenotype of the sensitive cells.

The failure of SW620 cells to undergo TRAIL-induced apoptosis was corrected by supplying cells with exogenous D-e-$C_6$-ceramide. Stereo-specificity was critical to achieve a synergistic response, because combination of TRAIL with the enantiomer L-e-$C_6$-ceramide was ineffective in inducing cell death. Lack of ceramide production has been associated with resistance to other death receptor ligands or chemotherapy (31, 32). For example, an increase in ceramide was detected in camptothecin-sensitive LNCaP prostate cancer cells but not chemoresistant PC3 cells. Exogenous ceramide was able to induce apoptosis in PC3 cells (32). Interestingly, TRAIL sensitivity in SW480 cells was not further enhanced by exogenous ceramide, suggesting that additional ceramide was not required for a maximal response. Because SW620 cells were derived from a metastasis of a primary tumor that contained TRAIL-sensitive cells (SW480) and TRAIL has been shown to exhibit antitumor activity *in vivo* (33), development of TRAIL resistance may reflect the necessity to acquire lesions in the TRAIL signaling pathway in order to escape the body's natural antitumor response. Defective TRAIL signaling in SW620 cells was not related to loss of TRAIL receptors or overexpression of antiapoptotic proteins,[3] and may result from lesions in ceramide metabolism or signaling.

The antiapoptotic protein cFLIP was investigated as one possible target of exogenous ceramide. Two previous studies clearly show the effect of ceramide on Akt inhibition and subsequent down-regulation of cFLIP. However, exogenous $C_6$-ceramide failed to affect either cFLIP isoform and pretreatment with ceramide did not result in sensitization to TRAIL, suggesting that exogenous ceramide enhances TRAIL sensitivity by a different mechanism. We found that TRAIL pretreatment was required to achieve synergy with ceramide, indicating a role for ceramide in promoting or amplifying TRAIL-induced apoptosis. Several groups have recently shown the importance of ceramide in lipid rafts, where it facilitates clustering of membrane rafts into ceramide-enriched platforms. The TNF-receptor has been localized to rafts (caveoli-like domains) and disruption of the rafts inhibited TNF-induced apoptosis (34). Cisplatin also increases ceramide levels leading to redistribution of Fas into lipids rafts, which contributed to cell death and sensitization to FasL-induced apoptosis (18). Exogenous $C_{16}$-ceramide can restore defective Fas-mediated apoptosis in acid sphingomyelinase-deficient cells by facilitating aggregation of Fas in the plasma membrane (35, 36). Thus, the mechanism by which exogenous $C_6$-ceramide enhances TRAIL-mediated apoptosis may be by increasing aggregation of TRAIL receptors, thereby eliciting a stronger apoptotic response.

TRAIL has received significant attention as a novel antitumor agent and a phase I clinical trial has recently been initiated. However, TRAIL as a single agent may not be effective against all cancers. Cancers that have developed TRAIL resistance may benefit from combination with agents that elevate intracellular ceramide levels or with ceramide itself. Difficulties in delivering $C_6$-ceramide have recently been overcome by encapsulation in liposomes (37). These $C_6$-ceramide-containing liposomes have been shown to be efficacious in breast cancer cells (37, 38). Combination of $C_6$-ceramide liposomes and TRAIL may be preferential to combining TRAIL with chemotherapeutic agents, particularly in tumors that have developed resistance to prior (chemo)therapies. Sustained sensitivity of SW620CT cells to ceramide/TRAIL treatment suggests that multiple rounds of combination therapy may be possible without developing resistance.

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[3] H. Gosnell and C. Voelkel-Johnson, unpublished data.

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