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Time Sequence of Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) and Cisplatin Treatment Is Responsible for a Complex Pattern of Synergistic Cytotoxicity

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Abstract The combination of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and cisplatin resulted in a greater cytotoxicity than could be accounted for by the addition of the cytotoxic effects of the agents alone. In this study, we hypothesized that the synergistic interaction between the two modalities can be changed when both the sequence and the time interval between the two treatments are varied. To test the hypothesis, human head-and-neck squamous-cell carcinoma (HNSCC)-6 cells were either pretreated with 0.01–0.5 μg/ml TRAIL for various times (0–24 h) followed by treatment with 5 μg/ml cisplatin or pretreated with 5 μg/ml cisplatin for various times (0–24 h) followed by treatment with 0.5 μg/ml TRAIL. In latter case, the synergistic effect was gradually increased when the time interval between the two treatments was increased. In former case, a maximal synergy occurred within 0–4 h of pretreatment with TRAIL. However, the synergistic effect was gradually decreased when the time interval between the two treatments was increased. Data from immunoblotting analysis reveal that a similar pattern emerged for the PARP cleavage and caspase activation. The synergistic effect is not associated with DR4, DR5, FADD, and $FLIP_L$. Interestingly, a complex pattern of synergistic interaction between TRAIL and cisplatin is related to the cleavage of $FLIP_S$. Although overexpression of $FLIP_S$ protected cells from $FLIP_S$ cleavage and apoptotic death, blockage of $FLIP_S$ cleavage by replacing Asp^{39} and Asp^{42} residues with alanine did not further enhance $FLIP_S$-mediated protection. Taken together, $FLIP_S$ cleavage reflects apoptotic damage, but it does not cause apoptosis. J. Cell. Biochem. 98: 1284–1295, 2006.

Key words: TRAIL; cisplatin; synergistic cytotoxicity; apoptosis; caspase

Head-and-neck squamous-cell carcinoma (HNSCC) is the sixth most common solid tumor in the developed world. It constitutes approximately 5% of all new cancers diagnosed annually in the United States [Catimel, 1996]. It accounted for approximately 40,000 new cases and 12,000 deaths in the United States in 1999. Cisplatin-based chemotherapy is widely used in the treatment for HNSCC. Although cisplatin is the most effective chemotherapeutic agent for HNSCC, randomized controlled trials do not indicate any consistent improvement in survival for patients receiving chemotherapy in addition to local treatment, as compared with patients receiving local treatment alone [Tannock and Browman, 1986]. Obviously, greater intervention will be required to significantly enhance HNSCC cancer therapy.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, APO-2L) is a type II integral membrane protein belonging to the tumor necrosis factor (TNF) family. TRAIL is a 281-amino acid protein, related most closely to Fas/APO-1 ligand. Like Fas ligand (FasL) and TNF, the C-terminal extracellular region of TRAIL (amino acids 114-281) exhibits a homotrimeric subunit structure [Pitti et al., 1996]. However, unlike FasL and TNF, it induces

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apoptosis in a variety of tumor cell lines more efficiently than in normal cells [Ashkenazi and Dixit, 1998]. The apoptotic signal induced by TRAIL is transduced by its binding to the death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5), which are members of the TNF receptor superfamily. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor-induced apoptosis. TRAIL also binds to TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), which act as decoy receptors by inhibiting TRAIL signaling [Degli-Esposti et al., 1997; Marsters et al., 1997; Pan et al., 1997; Sheridan et al., 1997; Walczak et al., 1997]. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif [Pan et al., 1997]. The relative resistance of normal cells to TRAIL has been explained by the presence of large numbers of the decoy receptors on normal cells [Gura, 1997]. Recently, this hypothesis has been challenged based on the results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines [Keane et al., 1999] and melanoma cell lines [Griffith et al., 1998]. This discrepancy indicates that other factors such as FLICE-inhibitory protein (FLIP), a death inhibitor [Griffith et al., 1998], are involved in the differential sensitivity to TRAIL.

Several studies have shown that cisplatin sensitizes TRAIL-induced cytotoxicity in a variety of tumor cells [Nagane et al., 2000; Lacour et al., 2001; Liu et al., 2001]. Similar results were also observed by combined treatment with TRAIL and ionizing radiation [Sheikh et al., 1998; Chinnaiyan et al., 2000; Gong and Almasan, 2000]. The mechanism of the synergy has been proposed to be via the upregulation of the TRAIL receptor DR5 [Chinnaiyan et al., 2000; Gong and Almasan, 2000; Nagane et al., 2000] or the downregulation of FLIP [Griffith et al., 1998]. However, recent studies show that DR5 expression does not correspond to the synergy in certain cell lines [Lacour et al., 2001; Liu et al., 2001]. Although this discrepancy needs to be clarified, the synergistic induction of apoptosis by the combination of TRAIL and cisplatin is still useful for chemotherapy. In this study, we focused on the effect of the time sequence of TRAIL and cisplatin treatment on the synergistic induction of cytotoxicity. We observed a complex pattern of synergistic cytotoxicity when the time interval between the two treatments was varied. Our results show that the synergistic cytotoxicity resulted from an increase in caspase activation. We observed that there was no change in the level of DR4, DR5, Fas-associated death domain (FADD), and $FLIP_L$ (a long form of FLIP). However, we observed a cleavage of $FLIP_S$ and this cleavage reflects apoptotic death.

MATERIALS AND METHODS

Cell Culture and Survival Determination

Human head-and-neck squamous-cell carcinoma HNSCC-6 cells were cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum (HyClone), 0.1 ng/ml human epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 2 mM L-glutamine, and 26 mM sodium bicarbonate for monolayer cell culture. The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO_2. Two days prior to the experiment, cells were plated into 60-mm dishes. The trypan blue exclusion assay was employed for survival determination. Cells were trypsinized, pelleted, and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min and examined.

Production of Recombinant TRAIL

A human TRAIL cDNA fragment (amino acids 114-281) obtained by RT-PCR was cloned into a pET-23d (Novagen) plasmid, and expressed protein was purified using the Ni-NTA His-Bind Resin Superflow according to the manufacturer's instructions (Qiagen).

Treatment With TRAIL and/or Cisplatin

Cells were replaced with fresh medium containing TRAIL and/or cisplatin (Bristol-Myers).

Morphological Evaluation

Approximately 5×10^5 cells were plated into 60-mm dishes overnight. Cells were treated with TRAIL and/or cisplatin and then analyzed by phase contrast microscopy for signs of apoptosis [Mac-Farlane et al., 1997].

Site-Directed Mutagenesis

The QuickChange site-directed mutagenesis kit (Stratagene) was used to make point mutations in $FLIP_S$ protein. Listed below are the various primers, which were used for converting two aspartic acid residues (Asp^{39}, Asp^{42}) to alanine in $FLIP_S$ to create one point mutants as well as a double mutant: sense primer oligonucleotides (5′-CCTAATGTCAGGGCCCTTCTGGATATTTTACG-3′) and antisense primer oligonucleotides (5′-CGTAAAATATCCAGAAGGGCCCTGACATTAGG-3′) for D39A; sense primer oligonucleotides (5′-GTCAGGGACCTTCTGGCTATTTTACGGGAAAG-3′) and antisense primer oligonucleotides (5′-CTTTCCCGTAAAATAGCCAGAAGGTCCCTGAC-3′) for D42A; sense primer oligonucleotides (5′-CCTAATGTCAGGGCCCTTCTGGCTATTTTACG-3′) and antisense primer oligonucleotides (5′-CGTAAAATAGCCAGAAGGGCCCTGACATTAGG-3′) for D39A/D42A. PCR reaction was prepared by adding 5 μl of 10× reaction buffer, 20 ng of dsDNA template (pAdlox-FLAG- $FLIP_S$), 125 ng of each sense primer, 125 ng of each antisense primer, 1 μl of dNTP mix, double-distilled water to a final volume of 50 μl, and 1 μl of *Pfu* Turbo DNA polymerase (2.5 U/μl). PCR was performed with 14 cycles (95°C for 30 s; 58°C for 1 min; 68°C for 7 min) with initial incubation at 95°C for 30 s. Following temperature cycling, the reaction was placed on ice for 2 min to cool the reaction. After PCR, 1 μl of *Dpn* I restriction enzyme (10 U/μl) was added directly to each amplification reaction which was incubated at 37°C for 1 h to digest the parental supercoiled dsDNA. The *Dpn* I-treated dsDNA was transformed into *Epicurian Coli* XL1-Blue supercompetent cells. Colonies were selected and each plasmid (pAdlox-FLAG-$FLIP_S$-ALLD; DLLA; ALLA) was sequenced using primer (5′-ATGTCTGCTGAAGTCATC-3′) to confirm mutation.

Shuttle Vector Construction

A FLAG-tagged FLIPs gene was isolated from pcDNA3-FLAG-FLIPs (kindly provided by Dr. P.M. Krammer, German Cancer Research Center, Heidelberg, Germany) by digesting with *Hind* III and *Sph* I and cloning into the *Hind* III and *Sph* I site of pAdlox shuttle vector [Hardy et al., 1997]. The complete shuttle vector was co-transfected into CRE8 cells with ψ5 viral genomic DNA for homologous recombination as described below.

Adenoviral Vector Construction

The adenovirus containing FLAG-tagged FLIPs was constructed by employing the Cre-lox recombinant system [Hardy et al., 1997]. The selective cell line CRE8 has a β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done by using Lipofectin™ Reagent (Invitrogen). Cells of 5×10^5 were split into 6-well plates 1 day before transfection. For the production of recombinant adenovirus, 2 μg of *Sfi* I/*Sca* I- digested Adlox/FLAG-FLIPs and 2 μg of ψ5 viral genomic DNA were cotransfected into CRE8 cells. The recombinant virus was generated by intermolecular homologous recombination between the shuttle vector and ψ5 viral DNA. The new virus had an intact packaging site and carried a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of FLAG-FLIPs to adenovirus was confirmed by Western blotting after infection of corresponding recombinant adenovirus into HNSCC-6 cells.

Protein Extracts and Polyacrylamide Gel Electrophoresis (PAGE)

Cells were lysed with 1 × Laemmli lysis buffer (2.4 M glycerol, 0.14 M Tris, pH 6.8, 0.21 M sodium dodecyl sulfate, 0.3 mM bromophenol blue) and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce). The samples were diluted with 1 × lysis buffer containing 1.28 M β-mercaptoethanol, and equal amounts of protein were loaded on 8–12% SDS–polyacrylamide gel. SDS–PAGE analysis was performed according to Laemmli [Laemmli, 1970] using a Hoefer gel apparatus.

Immunoblot Analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. The nitrocellulose membrane was blocked with 7.5% non-fat dry milk in PBS-Tween-20 (0.1%, v/v) at 4°C overnight. The membrane was incubated with anti-PARP (Biomol Research Laboratory), anti-caspase-8 (Upstate Biotechnology), anti-caspase-3 (Santa Cruz), anti-DR5 (Stressgen), or anti-FLIP antibody (Calbiochem) for 1 h. Horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. Immunoreactive

protein was visualized by the chemiluminescence protocol (ECL, Amersham).

RESULTS

The Time Sequence of TRAIL and Cisplatin: Effect of Pretreatment With TRAIL/Cisplatin on Induction of Cytotoxicity

The combination of TRAIL and cisplatin results in synergistic cytotoxicity in various cell lines [Nagane et al., 2000; Liu et al., 2001]. We examined whether the synergistic interaction between them would be changed when both the sequence and the time interval between the two treatments are varied. In order to study this possibility, HNSCC-6 cells were pretreated for various times with 0.5 μg/ml TRAIL followed by 5 μg/ml cisplatin for 24 h. Treatment with 5 μg/ml cisplatin alone for 24 h caused minimal cytotoxicity (panel c in Fig. 1A,B). Treatment with 0.5 μg/ml TRAIL alone for 24 h also showed minimal cytotoxicity, with approximately 10% cell killing (Fig. 1B). However, the cytotoxicity was significantly enhanced by the combined treatment with TRAIL and cisplatin (Fig. 1A,B); approximately 70% of cells were killed as observed in previous studies [Kim et al., 2003]. Most of the cells underwent apoptosis during TRAIL treatment in combination with cisplatin as shown by cell surface blebbing and formation of apoptotic bodies (Fig. 1A) and TUNEL assays (Fig. 1C). Interestingly, when the time interval between the two treatments was increased, a complex pattern of the survival curve was observed. A short period (4–8 h) of pretreatment with TRAIL enhanced the synergistic cytotoxicity (panels e and f in Fig. 1A,B). In contrast, a long period (24 h) of pretreatment with TRAIL reduced the synergistic cytotoxicity (panel h in Fig. 1A,B).

We next examined what happens when HNSCC-6 cells are first treated with cisplatin and then with TRAIL. Figure 2 shows that, unlike previous observations, the synergistic effect was gradually increased when the time interval between the two treatments was increased.

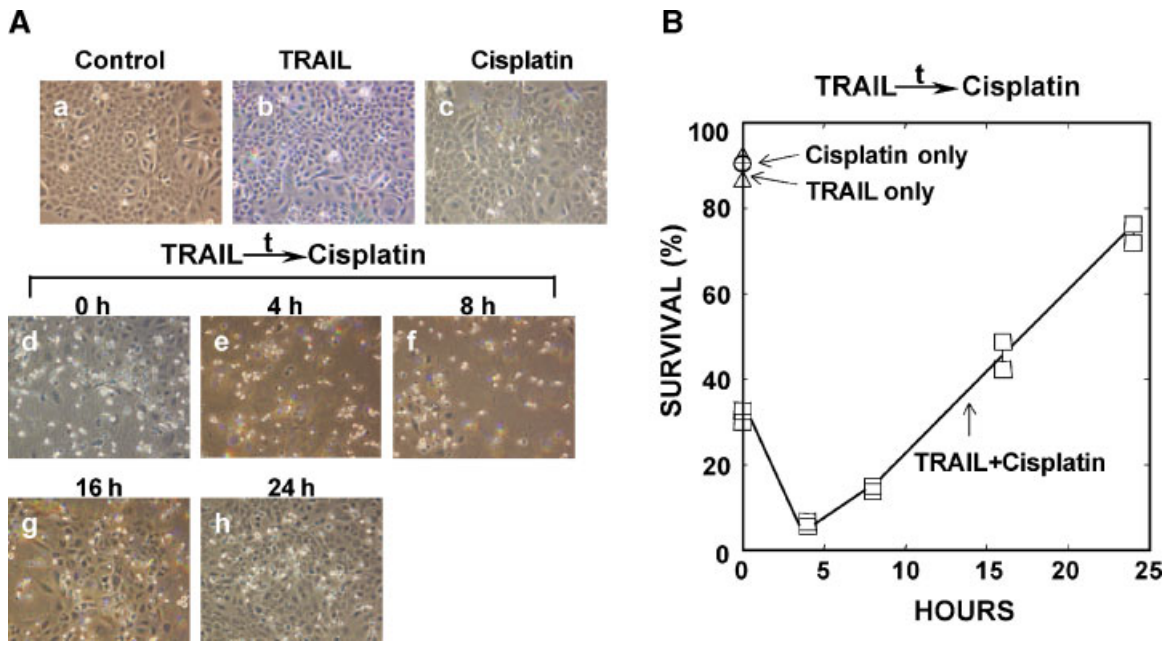


Fig. 1. Effect of pretreatment with TRAIL on cisplatin cytotoxicity in HNSCC-6 cells. **A panel**: Cells were pretreated with 0.5 μg/ml TRAIL for various times (0–24 h) followed by treatment with 5 μg/ml cisplatin for 24 h. Morphology was evaluated with a phase-contrast microscope. (a) Control, untreated control cells. (b) TRAIL, cells were treated with 0.5 μg/ml TRAIL alone for 24 h. (c) Cisplatin, cells were treated with 5 μg/ml cisplatin alone for 24 h. Various time intervals (0–24 h) between two treatments were shown in panels (d–h) in Figure 1A. **B panel**: Cells were pretreated with 0.5 μg/ml TRAIL for various times (0–24 h) followed by treatment with 5 μg/ml cisplatin for 24 h (TRAIL + Cisplatin). Survival was analyzed by trypan blue exclusion assay. Data represent two separate experiments. Cisplatin only, cells were treated with 5 μg/ml cisplatin alone for 24 h. TRAIL only, cells were treated with 0.5 μg/ml TRAIL alone for 24 h. **C panel**: Cells were pretreated with 0.5 μg/ml TRAIL for 4 h followed by treatment with 5 μg/ml cisplatin for 24 h (TRAIL + Cisplatin). Con, untreated control cells. TRAIL only, cells were treated with 0.5 μg/ml TRAIL alone for 4 h. Cisplatin only, cells were treated with 5 μg/ml cisplatin alone for 24 h. After treatment, apoptosis was detected by the TUNEL assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

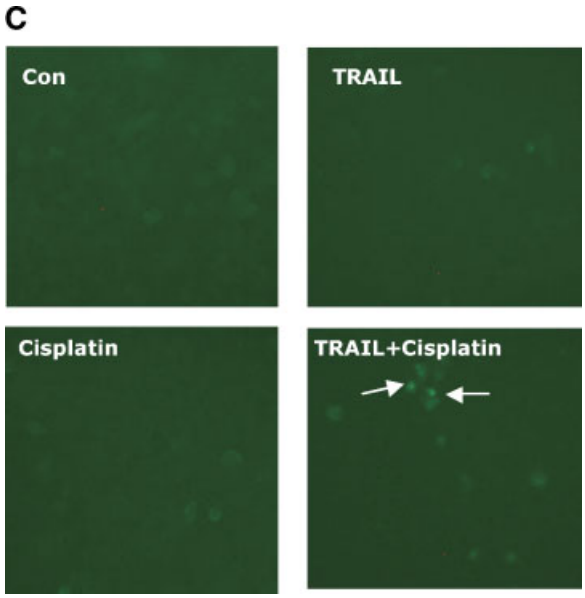
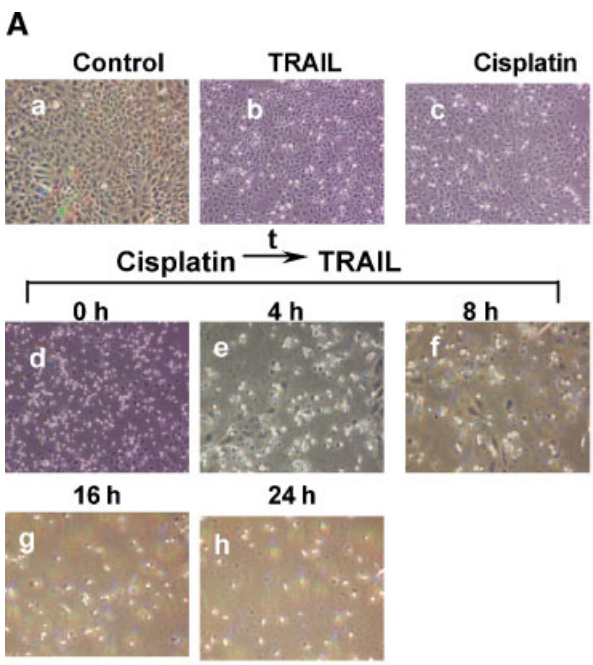


Fig. 1. (*Continued*)

Additional studies were designed to determine whether variation in times of pretreatment with TRAIL or cisplatin also changes the poly (ADP-ribose) polymerase (PARP) cleavage, the significant event of apoptosis. TRAIL (0.5 μg/ml) alone failed to induce PARP cleavage (lane 4 in Fig. 3C). Cisplatin (5 μg/ml) for 1 day, but not 2 days, caused no detectable amount of PARP cleavage (lane 2 in Fig. 3A,B vs. lane 3 in Fig. 3B). The combination of TRAIL and cisplatin treatment promoted PARP cleavage (lane 3 in Fig. 3A and lane 4 in Fig. 3B). PARP (116 kDa) was cleaved yielding a characteristic 85 kDa fragment. In case of pretreatment with 0.5 μg/m TRAIL, when the time interval between the two treatments was increased, PARP cleavage was promoted within 4 h (lane 4 in Fig. 3A) and gradually decreased. A significantly less amount of PARP was cleaved by 24 h after TRAIL treatment (lane 7 in Fig. 3A). Similar results were observed in low concentrations of TRAIL (0.01–0.2 μg/ml). PARP cleavage was gradually decreased when the time interval between the two treatments was increased (Fig. 4). However, in case of pretreatment with cisplatin, PARP cleavage was gradually increased when the time interval between the two treatments was increased (Fig. 3B).



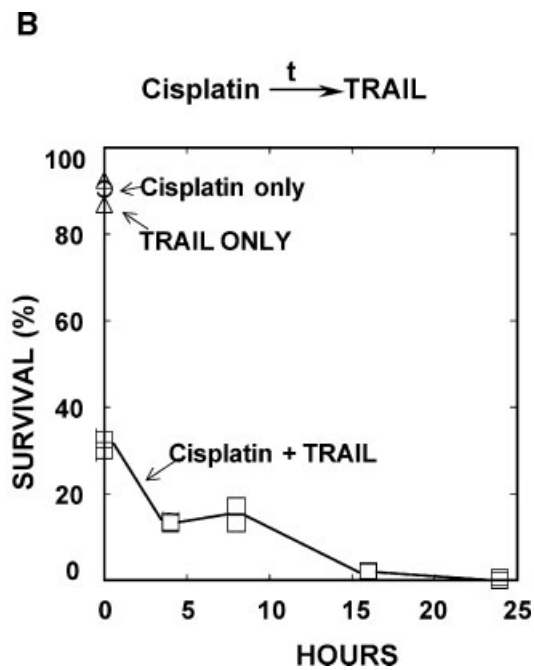
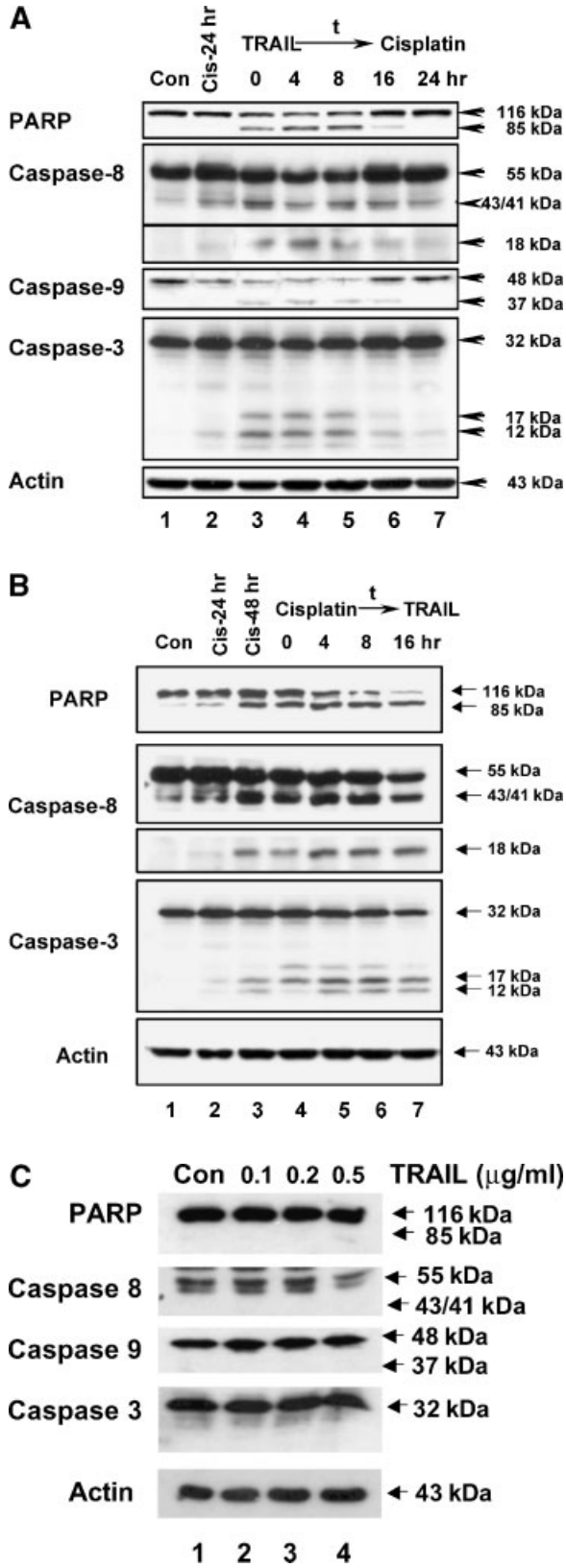


Fig. 2. Effect of postreatment with TRAIL on cisplatin cytotoxicity in HNSCC-6 cells. **A panel**: Cells were pretreated with 5 μg/ml cisplatin for various times (0–24 h) followed by treatment with 0.5 μg/ml TRAIL for 24 h. Morphology was evaluated with a phase-contrast microscope. (a) Control, untreated control cells. (b) TRAIL, cells were treated with 0.5 μg/ml TRAIL alone for 24 h. (c) Cisplatin, cells were treated with 5 μg/ml cisplatin alone for 24 h. Various time intervals (0–24 h) between two treatments were shown in panels (d–h) in Figure 2A. **B panel**: Cells were pretreated with 5 μg/ml cisplatin for various times (0–24 h) followed by treatment with 0.5 μg/ml TRAIL for 24 h (Cisplatin + TRAIL). Survival was analyzed by trypan blue exclusion assay. Data represent two separate experiments. Cisplatin only, cells were treated with 5 μg/ml cisplatin alone for 24 h. TRAIL only, cells were treated with 0.5 μg/ml TRAIL alone for 24 h. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



It is well known that apoptosis induced by TRAIL or cisplatin is characterized by the activation of caspases [Mariani et al., 1997; Kuwahara et al., 2000]. Thus, we further examined the effect of various time intervals between the two treatments on the activation of caspases, in particular, caspase-8 (FLICE) and caspase-3 (CPP32). Cisplatin (5 μg/ml) for 1 day caused a minimal activation of caspases (lane 2 in Fig. 3A,B). In contrast, 2 days of cisplatin treatment caused a significant activation of caspases (lane 3 in Fig. 3B); procaspase-8 (55 kDa), the precursor form of caspase-8, was cleaved to the intermediate (43 and 41 kDa) and active form (18 kDa), and procaspase-3 was cleaved to its signature forms (17 and 12 kDa). Combined treatment with 0.5 μg/ml TRAIL and 5 μg/ml cisplatin resulted in an increase in caspase-8 and -3 activation (lane 3 in Fig. 3A and lane 4 in Fig. 3B). These results indicate that synergistic induction of apoptosis by the combination of TRAIL and cisplatin is due to promotion of caspase activation. In case of pretreatment with TRAIL, the activation of caspases was enhanced by 4 h of pretreatment with TRAIL and then gradually reduced by increasing the time interval between the two treatments (Fig. 3A). These results consistently show that the combination of TRAIL and cisplatin synergistically induces apoptotic death and an increase in pretreatment time with TRAIL promotes the synergistic cytotoxicity within a short period and eventually decreases the synergistic effect. However, in case of pretreatment with cisplatin, the activation of

Fig. 3. Effect of time sequence of TRAIL and cisplatin treatment on proteolytic cleavage of PARP and activation of caspases. HNSCC-6 cells were treated with (**A**) 0.5 μg/ml TRAIL for various times (0–24 h; **lanes 3–7**) followed by treatment with 5 μg/ml cisplatin for 24 h, (**B**) 5 μg/ml cisplatin for various times (0–16 h; **lanes 4–7**) followed by treatment with 0.5 μg/ml TRAIL for 24 h, or (**C**) various concentrations (0.1–0.5 μg/ml) of TRAIL for 4 h. Cells were treated with 5 μg/ml cisplatin alone for 24 h (**lane 2** in Fig. 3A,B) or 48 h (**lane 3** in Fig. 3B). Lysates from equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted. PARP: The upper band indicates 116 kDa PARP whereas the lower band indicates the 85 kDa apoptosis-related cleavage fragment. Caspase-8: Antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (43 kDa and 41 kDa), and active subunit (18 kDa). Caspase-9: Anti-caspase-9 antibody detects both inactive form (48 kDa) and intermediate form (37 kDa). Caspase-3: Anti-caspase-3 antibody detects both inactive form (32 kDa) and active forms (17 kDa and 12 kDa). Actin: Actin was used to confirm the equal amount of proteins loaded in each lane. Con: Untreated control cells.

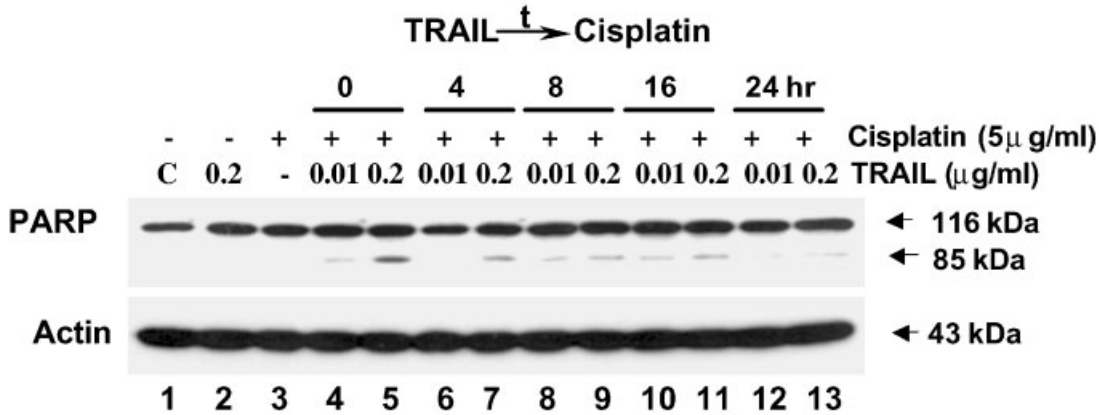


Fig. 4. Proteolytic cleavage of PARP by treatment with various concentrations of TRAIL prior to cisplatin. HNSCC-6 cells were treated with TRAIL alone (0.2 μg/ml; **lane 2**), cisplatin alone (5 μg/ml; **lane 3**), or pretreated with TRAIL (0.01–0.2 μg/ml) for various times (0–24 h; **lanes 4–13**) followed by treatment with 5 μg/ml cisplatin for 24 h. Lysates from equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted with anti-PARP or anti-actin antibody.

caspases were continuously increased when the time interval between the two treatments was increased (Fig. 3B).

Intracellular Levels of Apoptosis-Associated Proteins After Combined Treatment With TRAIL and Cisplatin

Previous studies have shown that genotoxic agents such as chemotherapeutic agents [Griffith et al., 1998; Keane et al., 1999; Nagane et al., 2000] and ionizing radiation [Chinnaiyan et al., 2000] can enhance TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP [Griffith et al., 1998] or increasing DR5 gene expression in response to genotoxic stress [Sheikh et al., 1998; Chinnaiyan et al., 2000; Nagane et al., 2000]. To investigate whether these proteins are associated with the enhancement of apoptosis by TRAIL in combination with cisplatin, we examined the intracellular levels of DR4, DR5, FADD, and FLIP (Fig. 5) by Western blot. The levels of DR4, DR5, and FADD were not changed in HNSCC-6 cells treated with TRAIL and/or cisplatin relative to untreated control cells (Figs. 5A,B and 6A,B).

Although the FLIP level in HNSCC-6 cells exposed to TRAIL or cisplatin for 24 h was not changed (Figs. 5C and 6B), cisplatin in combination with TRAIL resulted in a decrease in

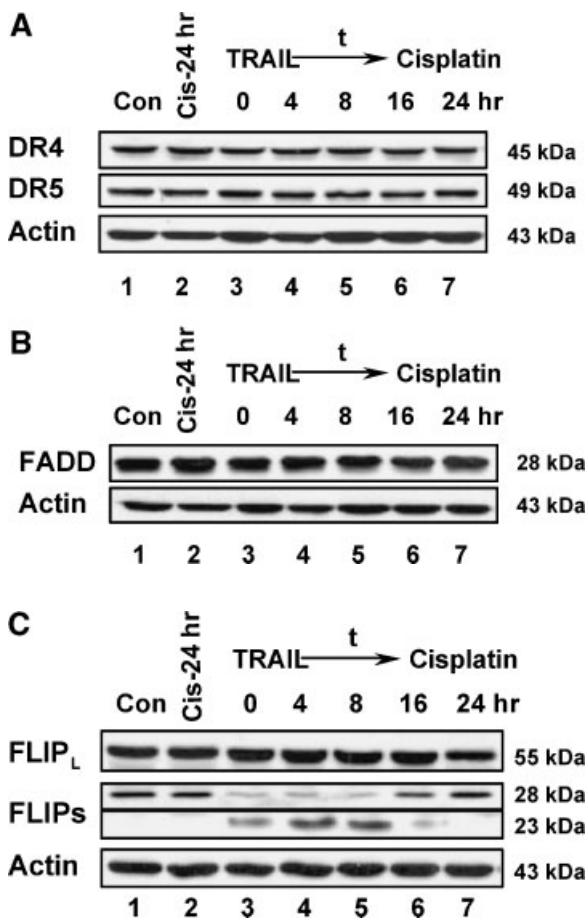


Fig. 5. Intracellular levels of (A) DR4, DR5, (B) FADD, (C) $FLIP_L$, and $FLIP_S$ during treatment with TRAIL and cisplatin. HNSCC-6 cells were treated with cisplatin alone (5 μg/ml; **lane 2**), or pretreated with 0.5 μg/ml TRAIL for various times (0–24 h; **lanes 3–7**) followed by treatment with 5 μg/ml cisplatin for 24 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted by using a polyclonal antibody against DR4, DR5, FADD, $FLIP_L$, and $FLIP_S$. Actin was shown as an internal standard.

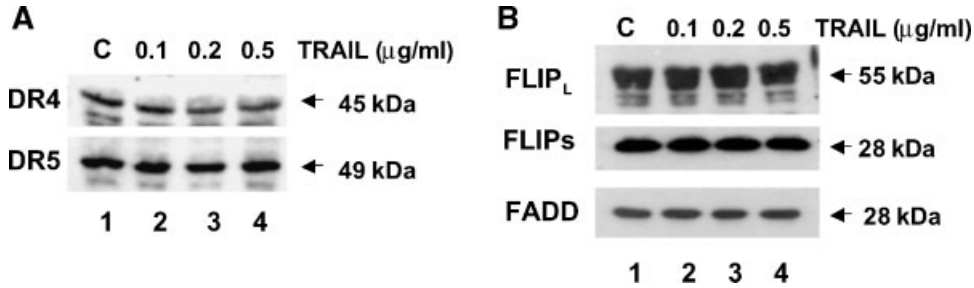


Fig. 6. Intracellular levels of (A) DR4, DR5, (B) FADD, $FLIP_L$, and $FLIP_S$ during treatment with TRAIL and cisplatin. HNSCC-6 cells were treated with various concentrations of TRAIL (0.1–0.5 μg/ml) for 4 h. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted by using a polyclonal antibody against DR4, DR5, FADD, $FLIP_L$, and $FLIP_S$. Actin was shown as an internal standard.

$FLIP_S$ level (Fig. 5C). The reduction of $FLIP_S$ was due to its cleavage. The active form of $FLIP_S$ (28 kDa) was cleaved to the inactive form (23 kDa). These data demonstrate that the reduction of $FLIP_S$ level by TRAIL in combination with cisplatin correlated with an increase in apoptosis in HNSCC-6 cells.

Role of Caspase-3 in the Cleavage of $FLIP_S$

To investigate whether caspase-3 is involved in the cleavage of the precursor form of $FLIP_S$, HNSCC-6 cells were pretreated with Z-DEVD-FMK, a caspase-3 inhibitor, prior to treatment with TRAIL followed by cisplatin. Figure 7 shows that 4 h pretreatment with TRAIL followed by cisplatin-induced PARP cleavage, caspase-3 activation as well as $FLIP_S$ cleavage (lane 4 in Fig. 7). Pretreatment with Z-DEVD-FMK completely inhibited them (lane 5 in Fig. 7). These results suggest that caspase-3 is involved in the reduction of $FLIP_S$ level. Inhibition of caspase-3 activation also prevented PARP cleavage and apoptotic death (data not shown). Taken together, caspase-3 is involved in the cleavage of $FLIP_S$ and the cleavage of $FLIP_S$ may reflect apoptotic death.

Overexpression of FLIPs Protects Cells From Cleavage of FLIPs During Treatment With TRAIL and Cisplatin

Since $FLIP_S$ functions as an antiapoptotic molecule, we hypothesized that the cleavage of $FLIP_S$ may cause it to lose its biological function. To test this hypothesis, we investigated whether overexpression of $FLIP_S$ inhibits $FLIP_S$ cleavage during treatment with TRAIL and cisplatin and whether inhibition of $FLIP_S$ cleavage protects cells from apoptotic death. HNSCC-6 cells were infected with control adenoviral vector (Mock) or adenoviral vector containing wild-type FLAG-tagged $FLIP_S$ cDNA (Ad.FLAG-FLP_S, wild) (Fig. 8B). Infected cells were pretreated with 1 μg/ml TRAIL for 4 h prior to treatment with 10 μg/ml cisplatin. Figure 8B shows that overexpression of $FLIP_S$ prevented $FLIP_S$ cleavage during treatment with TRAIL and cisplatin. To examine whether $FLIP_S$ cleavage is responsible for apoptotic death during treatment with TRAIL and cisplatin, we employed site-directed mutagenesis techniques to create one point mutants

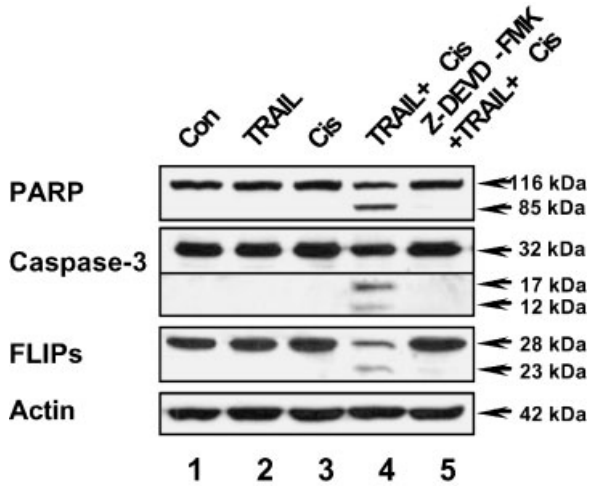


Fig. 7. Effect of caspase-3 inhibitor on treatment with TRAIL and cisplatin-induced PARP cleavage, caspase-3 activation, and $FLIP_S$ cleavage in HNSCC-6 cells. Cells were pretreated with 20 μM Z-DEVD-FMK for 30 min and then treated with 0.5 μg/ml TRAIL for 4 h prior to treatment with 5 μg/ml cisplatin for 24 h (Z-DEVD-FMK → TRAIL → Cisplatin; **lane 5**). Cells were pretreated with 0.5 μg/ml TRAIL for 4 h prior to treatment with 5 μg/ml cisplatin for 24 h (TRAIL → Cisplatin; **lane 4**). TRAIL, cells were treated with 0.5 μg/ml TRAIL alone for 28 h. Cis, cells were treated with 5 μg/ml cisplatin alone for 24 h. Con, untreated control cells. Lysates containing equal amounts of protein (20 μg) were separated by SDS–PAGE and immunoblotted as described in Figures 2 and 4.

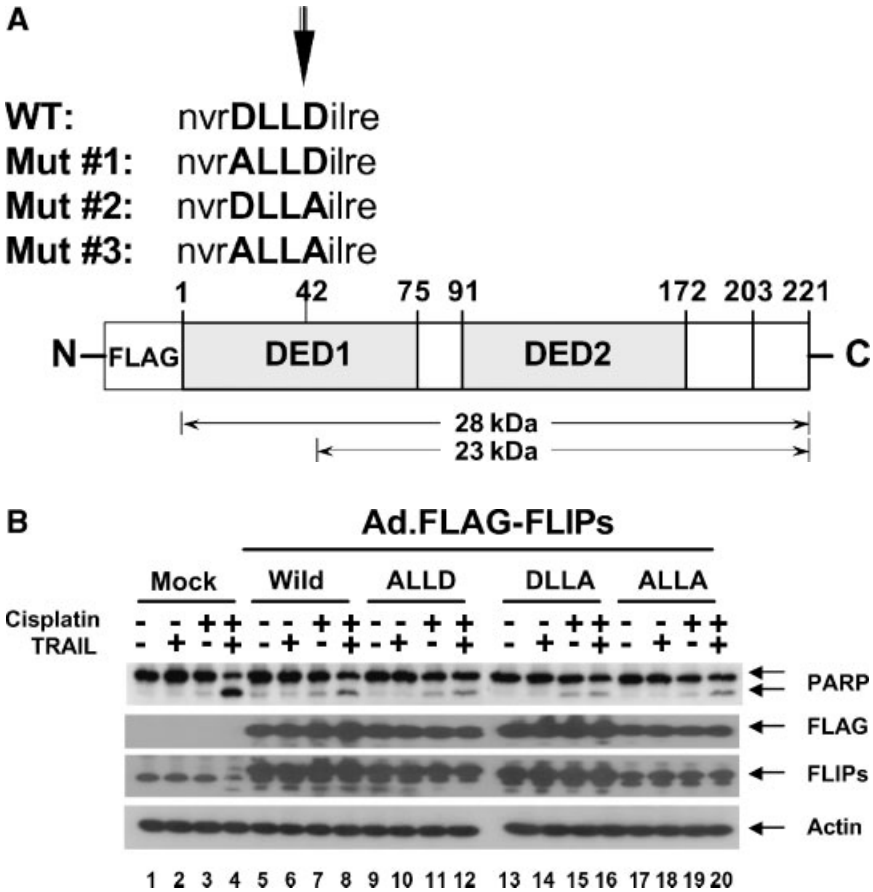


Fig. 8. Schematic diagram of wild- and mutant-type $FLIP_S$ (**A**) and their effect on apoptotic death (**B**). A: An arrow shows a putative cleavage site at Asp^{42}-Ile^{43} by caspase-3. Asp^{39} and Asp^{42} residues are replaced with alanine. B: HNSCC-6 cells were infected with adenoviral vectors containing FLAG-tagged wild- or mutant-type $FLIP_S$ (Ad/FLAG-$FLIP_S$) at a multiplicity of infection of 50. After 24 h of incubation, cells were pretreated with 1 μg/ml TRAIL for 4 h and treated with 10 μg/ml cisplatin for 24 h. Cell lysates were immunoblotted with anti-PARP, anti-FLAG, anti- $FLIP_S$, or anti-actin antibody. Actin is shown as an internal standard.

($^{39}DLLD^{42} \rightarrow {}^{39}ALLD^{42}$ or $^{39}DLLA^{42}$) or a double mutant ($^{39}DLLD^{42} \rightarrow {}^{39}ALLA^{42}$) at a putative cleavage site by caspase-3 [Kim et al., 2003; Fig. 8A]. HNSCC-6 cells were infected with adenoviral vector containing wild-type $FLIP_S$ or mutant-type $FLIP_S$. Cells were pretreated with 1 μg/ml TRAIL for 4 h followed by 10 μg/ml cisplatin for 24 h. Figure 8B shows that wild-type $FLIP_S$ as well as mutant-type $FLIP_S$ effectively prevented PARP cleavage during treatment with TRAIL and cisplatin. These results suggest that the cleavage of $FLIP_S$ reflects apoptotic death. It is not responsible for apoptosis.

DISCUSSION

In this study, we observed that the synergistic interaction between TRAIL and cisplatin is dependent upon the time interval between the two treatments. A maximal synergy was observed when cells were pretreated with TRAIL for 0–4 h prior to cisplatin treatment. Interestingly, this synergistic effect was gradually reduced when the time interval was further increased. In contrast, the synergistic effect was continuously increased when cells were pretreated with cisplatin followed by treatment with TRAIL. This difference is probably due to differential apoptotic pathways by different agents. We previously observed that TRAIL-induced caspase-3 activation and apoptosis are mediated through two different apoptotic pathways, mitochondria-dependent and mitochondria-independent [Lee et al., 2001a]. Recent studies demonstrated that the FADD is required for TRAIL-induced apoptosis [Kischkel et al., 2000; Kuang et al., 2000; Sprick et al.,

2000]. TRAIL triggers apoptosis by recruiting the apoptosis initiator procaspase-8 through the adaptor FADD [Bodmer et al., 2000; Kischkel et al., 2000]. Caspase-8 can directly activate downstream effector caspases including procaspase-3, -6, and-7 [Cohen, 1997]. Caspase-8 also cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome *c* release [Li et al., 1998; Schendel et al., 1999]. Cytochrome *c* in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9. Caspase-9 cleaves and activates procaspase-3 [Slee et al., 1999]. Interestingly, TRAIL-induced cytotoxicity is not significantly inhibited by overexpression of Bcl-2, an antiapoptotic molecule [Gazitt et al., 1999; Walczak et al., 2000; Lee et al., 2001a]. These results suggest that TRAIL-induced apoptosis is primarily dependent upon a mitochondria-independent pathway. In chemical-induced apoptosis, unlike TRAIL-mediated apoptosis, cytochrome *c* release is caspase-independent and is not mediated by cleavage of Bid [Sun et al., 1999]. Recent studies reveal that a specific inhibitor of caspase-9 almost completely inhibits cisplatin-induced apoptotic death [Kuwahara et al., 2000]. These results indicate cisplatin-induced apoptosis is mediated through the mitochondria-dependent pathway. A fundamental question which remains unanswered is how a complex pattern of synergistic cytotoxicity occurred when the time interval between TRAIL and cisplatin treatment or the time interval between sequential events was varied. The complex pattern is probably due to the activation of different types of apoptotic pathways: receptor-mediated versus non-receptor-mediated. We previously observed that receptor-mediated apoptosis occurs within a few hours [Lee et al., 2001b]. In contrast, non-receptor-mediated apoptotic death usually takes a longer period, perhaps from 1 to 2 days (lane 3 in Fig. 3B). In case of pretreatment with TRAIL, it is possible that cross-talk between a receptor-mediated apoptotic pathway and a non-receptor-mediated apoptotic pathway occurs within several hours and gradually becomes diminished. However, in case of pretreatment with cisplatin, a non-receptor-mediated apoptotic pathway may amplify a receptor-mediated apoptotic pathway through activating p38 mitogen-activated protein kinase (MAPK) and c-Jun NH2-terminal kinase (JNK) pathways [Hsieh and Nguyen, 2005]. In fact, previous studies showed that JNK activity is triggered by cisplatin-induced DNA damage and activation of JNK plays an important role in mediating cisplatin induced apoptosis [Bulmer et al., 2005; Li et al., 2005]. Alternatively, recent studies demonstrated that cisplatin induces endoplasmic reticulum stress which results in activation of calpain and subsequently promotes Bid cleavage [Mandic et al., 2002, 2003]. Cisplatin also induces calpain-independent Bak modulation [Mandic et al., 2002]. Cross-talk between calpain-associated signals and activation of caspases may be responsible for the synergistic effect of cisplatin in combination with TRAIL. Although this possibility needs to be further investigated, we believe that this model will provide a framework for future studies.

Previous studies have shown that several c-FLIP splice variants exist on the mRNA level, but two endogenous forms, $FLIP_L$ and $FLIP_S$, are detected on the protein level [Irmler et al., 1997; Shu et al., 1997]. The role of c-FLIP in apoptosis signaling has been controversial. Some reports have described it as an antiapoptotic molecule [Goltsev et al., 1997; Tschopp et al., 1998; Scaffidi et al., 1999] and others as a proapoptotic molecule [Han et al., 1997; Inohara et al., 1997]. Our studies reveal that $FLIP_S$ has an antiapoptotic function (Fig. 8). Recent studies show that $FLIP_L$ and $FLIP_S$ prevent caspase-8 activation at different levels of procaspase-8 processing at the death-inducing signaling complex (DISC). $FLIP_S$ completely inhibits cleavage of procaspase-8, whereas $FLIP_L$ inhibits the second cleavage step of procaspase-8 [Krueger et al., 2001]. Figure 7 shows that the active form of $FLIP_S$ (28 kDa) was cleaved to the inactive form (23 kDa). Z-DEVD-FMK, a caspase-3 inhibitor, inhibits $FLIP_S$ cleavage during treatment with TRAIL followed by cisplatin. These results suggest that caspase-3 is involved in the cleavage of $FLIP_S$. Figures 3 and 5 clearly show that the cleavage of $FLIP_S$ is coordinated with the activation of caspase-3. We previous reported that overexpression of $FLIP_S$ protects cells from apoptotic death and cleavage of $FLIP_S$ is one of the facilitating factors for TRAIL-induced apoptosis. In this study, however, we demonstrated that blockage of $FLIP_S$ cleavage by replacing Asp^{39} and Asp^{42} residues with alanine did not further enhance $FLIP_S$-mediated protection. Thus, these results suggest that $FLIP_S$ cleavage

reflects apoptotic damage, but it does not cause apoptosis.

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