Role of Antiapoptotic Proteins in Tumor Necrosis Factor-related Apoptosis-inducing Ligand and Cisplatin-augmented Apoptosis

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INTRODUCTION

HNSCC is the sixth most common solid tumor worldwide, accounting for about 5% of all new cancers diagnosed annually in the United States (1). Although recent advances in management with a multidisciplinary approach including chemotherapy in combination with radiotherapy or surgery result in improved local and regional disease control, the overall survival rate has not improved much during the last decade. Obviously, greater intervention will be required to significantly enhance HNSCC cancer therapy.

Cisplatin is indicated in various combinations of chemotherapeutic regimens for ovarian, head and neck, bladder, cervical, and other neoplasms. In addition to its toxic side effects, a major limitation of cisplatin chemotherapy is drug resistance. The dose scale necessary to overcome even a small increase in cellular resistance can cause severe cytotoxicity. Understanding the molecular basis of cisplatin-mediated apoptosis could significantly improve clinical protocols.

TRAIL/APO-2 ligand is a type II integral membrane protein belonging to the TNF family. TRAIL is a 281-amino acid protein, related most closely to Fas/APO-1 ligand. Like Fas ligand and TNF, the COOH-terminal extracellular region of TRAIL (amino acids 114–281) exhibits a homotrimeric subunit structure (2). It induces apoptosis in a variety of tumor cell lines more efficiently than normal cells (3). The apoptotic signal induced by TRAIL is transduced by its binding to the DRs, 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 DcRs by inhibiting TRAIL signaling (4–10). 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 (4). The relative resistance of normal cells to TRAIL has been explained by the presence of large numbers of the DcRs on normal cells (11, 12). Recently, this hypothesis has been challenged, based on results showing poor correlations between DR4, DR5, and DcR1 expression and sensitivity to TRAIL-induced apoptosis in normal and cancerous breast cell lines (13). This discrepancy indicates that other factors such as death receptor expression and other death ligands may also play a role in cancer resistance to TRAIL.

FLIP (Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein) is expressed as long (FLIP-L) and short (FLIP-S) forms (2). The short form of FLIP-S contains the death domain required for receptor-mediated apoptosis, whereas the long form is only expressed in response to TNF-α and TRAIL (3) and can suppress TRAIL-induced apoptosis by competitive binding to the corresponding receptor. Benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK), a caspase-3 inhibitor, blocked the cleavage of FLIPS and caspase-3 activation. Overexpression of FLIPs protected cells from apoptotic death and FLIPS cleavage during treatment with TRAIL in combination with cisplatin.

Conclusions: These results suggest that caspase-3 is responsible for FLIPS cleavage, and the cleavage of FLIPS is one of facilitating factors for TRAIL-induced apoptotic death.

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3 The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; DR, death receptor; DED, death effector domain; DR, death receptor; EGFP, enhanced green fluorescent protein; FLIP, Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FLIPs, long form of FLIP; FLIPS, short form of FLIP; DISC, death-inducing signaling complex; Z-DEVD-FMK, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone.
inhibitors (FLIP, Fas-associated protein-1, or inhibitor of apoptosis) are also involved in the differential sensitivity to TRAIL.

Several researchers have also shown that genotoxic agents such as chemotherapeutic agents (13–15) and ionizing radiation (16, 17) can sensitize cells to killing by TRAIL. The synergistic effect is probably due to up-regulation of DR5 expression (15, 16) or down-regulation of FLIP expression (14). In this study, we examined whether the synergistic induction of apoptosis by treatment with TRAIL and cisplatin in HN5SC is associated with modulation of TRAIL receptors or FLIP. Our results show that TRAIL in combination with cisplatin did not alter the level of DR4, DR5, DcR2, and a FLIP<sub>S</sub>. However, it resulted in cleavage of FLIP<sub>S</sub>. Our results also suggest that the cleavage of FLIP<sub>S</sub> is mediated through activation of caspase-3.

MATERIALS AND METHODS

Cell Culture and Survival Determination. Human HN5SC cells (HN5SC-6 and HN5SC-30) were cultured in DMEM/Ham’s F-12 supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 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<sub>2</sub>. Two C humidification culture. The dishes containing cells were kept in a 37°C humidified incubator with a mixture of 95% air and 5% CO<sub>2</sub>. Two days before the experiment, cells were plated into 60-mm dishes. The trypan blue exclusion assay was used 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 PBS solution. 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 reverse transcription-PCR was cloned into a pET-23d (Novagen, Madison, WI) plasmid by Dr. D. W. Seol (18), and expressed protein was purified from E. coli BL21 (Stratagene, La Jolla, CA).

Treatment with TRAIL and/or Cisplatin. Cells were replaced with fresh medium containing TRAIL and/or cisplatin (Bristol-Myers, Evansville, IN).

Morphological Evaluation. Approximately 5 × 10<sup>5</sup> 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 (19).

Shuttle Vector Construction. A FLAG-tagged FLIP<sub>S</sub> gene was isolated from pcDNA3-FLAG-FLIP<sub>S</sub> (kindly provided by Dr. P. M. Krammer; German Cancer Research Center, Heidelberg, Germany) by digestion with HindIII and SphI and cloned into the HindIII and SphI site of pAdlox shuttle vector (20). The complete shuttle vector was cotransfected into CRE8 cells with ψ5 viral genomic DNA for homologous recombination as described below.

Adenoviral Vector Construction. The adenovirus containing FLAG-tagged FLIP<sub>S</sub> was constructed by using the Crelox recombinase system (20). The selective cell line CRE8 has a β-actin-based expression cassette driving a Cre recombinase gene with an NH<sub>2</sub>-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done by using Lipofectin Reagent (Invitrogen, Carlsbad, CA). Cells (5 × 10<sup>5</sup>) were split into a 6-well plate 1 day before transfection. For the production of recombinant adenovirus, 2 μg of SfiI/ScaI-digested Adlox/FLAG-FLIP<sub>S</sub> 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. A new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed, and purified. The insertion of FLAG-FLIP<sub>S</sub> to adenovirus was confirmed by Western blotting after infection of corresponding recombinant adenovirus into HN5SC-6 cells.

Protein Extracts and PAGE. Cells were lysed with 1× Laemmli lysis buffer [2.4 M glycerol, 0.14 M Tris (pH 6.8), 0.21 M SDS, and 0.3 mM b-mercaptoethanol] and boiled for 10 min. Protein content was measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). The samples were diluted with 1× lysis buffer containing 1.28 M β-mercaptoethanol, and an equal amount of protein was loaded on 8–12% SDS-polyacrylamide gels. SDS-PAGE analysis was performed according to Laemmli (21) 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% nonfat dry milk in PBS-Tween 20 (0.1%, v/v) at 4°C overnight. The membrane was incubated with anti-PARP (Biomial Research Laboratory, Plymouth Meeting, PA), anti-caspase-8 (Upstate Biotechnology, Lake Placid, NY), anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-DR5 (Stressgen, Victoria, British Columbia, Canada), anti-DR4 (Upstate Biotechnology), anti-DcR2 (Stressgen), or anti-FLIP antibody (Calbiochem, Darmstadt, Germany) for 1 h. Horseradish peroxidase-conjugated antirabbit or antimouse IgG was used as the secondary antibody. Immunoreactive protein was visualized by the chemiluminescence protocol (ECL; Amersham, Arlington Heights, IL).

Flow Cytometry. Cells were pelleted and washed with fluorescence-activated cell-sorting buffer (PBS, 1% BSA, and 0.1% sodium azide). Cells were incubated with optimal concentrations of four recently developed phycoerythrin-conjugated monoclonal antibodies against extracellular domains of functional (DR4 and DR5) and decoy (DcR1 and DcR2) Apo2 ligand/TRAIL receptors (all from eBioscience, San Diego, CA) for 30 min at 4°C. Matched isotype control IgG antibodies were included. Analysis was performed using the FACScan flow cytometer, and results were analyzed with CellQuest software (both from Becton Dickinson Immunocytometry Systems, San Jose, CA).

RESULTS

Synergistic Induction of Cytotoxicity by the Combination of TRAIL and Cisplatin. The effect of TRAIL on cell death in conjunction with cisplatin was assessed in human HN5SC-6 cell line. Fig. 1A shows that treatment with 0.1–1.0 μg/ml TRAIL alone or 1–10 μg/ml cisplatin alone for 24 h caused minimal cytotoxicity: approximately 10–30% of cells were killed. However, the cytotoxicity was significantly enhanced by the combination treatment (Fig. 1A, TRAIL + Cisplatin). For example, more than 70% of cells were killed when cells were exposed to 0.5 μg/ml TRAIL in combination
with 5 μg/ml cisplatin. These observations were consistent with morphological features (Fig. 1B). Most cells underwent apoptosis during TRAIL treatment in combination with cisplatin (Fig. 1B, bottom right panel) as shown by cell surface blebbing and formation of apoptotic bodies. Additional studies were designed to determine whether the combination of TRAIL and cisplatin enhances PARP cleavage, the significant event of apoptosis, in HNSCC-6 cells.

A Combination of TRAIL and Cisplatin Treatment Promotes the Cleavage of PARP and the Activation of Caspases. It is well known that apoptosis induced by TRAIL or cisplatin is characterized by the cleavage of PARP and activation of caspases (22). Thus, we examined the effect of the combination of TRAIL and cisplatin on the cleavage of PARP and the activation of caspases, in particular, caspase-8 (Fas-associated death domain-like interleukin-1β-converting enzyme) and caspase-3 (CPP32) activation. HNSCC-6 cells were treated with TRAIL (0.1–1 μg/ml), cisplatin (1–10 μg/ml), or a combination of TRAIL (0.1–1 μg/ml) and cisplatin (1–10 μg/ml) for 24 h and then harvested. Lysates from equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. A, the top band indicates 116-kDa PARP, whereas the bottom band indicates the 85-kDa apoptosis-related cleavage fragment. B, antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (43 and 41 kDa), and active subunit (18 kDa). Anti-caspase-3 antibody detects both the inactive form (32 kDa) and active form (17 and 12 kDa). Actin, actin was used to confirm the equal amount of proteins loaded in each lane.

Fig. 2 Induction of PARP cleavage (A) and activation of caspases (B) after treatment with a combination of TRAIL and cisplatin. HNSCC-6 cells were treated with TRAIL alone (0.1–1 μg/ml), cisplatin alone (1–10 μg/ml), or a combination of TRAIL (0.1–1 μg/ml) and cisplatin (1–10 μg/ml) for 24 h and then analyzed. Lysates from equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. A, the top band indicates 116-kDa PARP, whereas the bottom band indicates the 85-kDa apoptosis-related cleavage fragment. B, antibody against caspase-8 detects inactive form (55 kDa), cleaved intermediates (43 and 41 kDa), and active subunit (18 kDa). Anti-caspase-3 antibody detects both the inactive form (32 kDa) and active form (17 and 12 kDa). Actin, actin was used to confirm the equal amount of proteins loaded in each lane.
cisplatin alone, or the combination for 24 h. Fig. 3 shows that the combination of TRAIL and cisplatin synergistically induces cell death (Fig. 3A), PARP cleavage (Fig. 3B), and activation of caspases (Fig. 3B) in HNSCC-30 cells. Our results were similar to those in the HNSCC-6 cell line. Thus, these results suggest that the synergistic effect can be generalized regardless of p53 status.

**Total Cellular Levels of TRAIL Receptors, Surface Expression of Functional TRAIL Receptors, and the Level of FLIP<sub>S</sub> during Combined Treatment with TRAIL and Cisplatin.** Previous studies have shown that genotoxic agents such as chemotherapeutic agents (13–15) and ionizing radiation (16) can sensitize TRAIL-induced cytotoxicity by decreasing intracellular levels of FLIP (14) or increasing DR5 gene expression in response to genotoxic stress (15, 16, 24). 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, DcR2, and FLIP by Western blot (Fig. 4, A and C). The surface expression of TRAIL receptors was examined by flow cytometry (Fig. 4B). The total cellular levels of DR4, DR5, DcR2, and FLIP, and the surface expression of DcR1, DcR2, and DR4 were not significantly changed in HNSCC-6 cells treated with TRAIL and/or cisplatin relative to untreated control cells (Fig. 4). Interestingly, Fig. 4B shows that cisplatin induced surface expression of DR5. However, combination of cisplatin and TRAIL did not enhance the DR5 expression. Unlike DR5, cisplatin alone (10 μg/ml) or in combination with TRAIL resulted in a decrease in the level of 28-kDa FLIPS, whereas there was an increase in the level of the fragment form of FLIPS (23 kDa; Fig. 4C).

**Caspase-3 Is Involved in the Cleavage of FLIP<sub>S</sub>.** To investigate whether caspase-3 is involved in the cleavage of the precursor form of FLIP<sub>S</sub>, HNSCC-6 cells were pretreated with Z-DEVD-FMK, a caspase-3 inhibitor, before treatment with TRAIL and cisplatin. Fig. 5 shows that TRAIL in combination with cisplatin-induced caspase-3 activation as well as the cleavage of FLIP<sub>S</sub> were inhibited by pretreatment with Z-DEVD-FMK. These results suggest that caspase-3 is involved in the reduction of FLIP<sub>S</sub> level. Inhibition of caspase-3 activation also blocked the cytotoxicity of combined TRAIL and cisplatin (data not shown). Taken together, cell death by combination of TRAIL and cisplatin is due to caspase-3-mediated apoptosis, and activation of caspase-3 is involved in the cleavage of FLIP<sub>S</sub>.

**Overexpression of FLIP<sub>L</sub> Protects Cells from Cell Death and Cleavage of FLIP<sub>S</sub> during Treatment with TRAIL and Cisplatin.** Recent studies show that FLIP<sub>L</sub> and FLIP<sub>S</sub> prevent caspase-8 activation at different levels of procaspase-8 processing at the DISC. FLIP<sub>S</sub> completely inhibits cleavage of procaspase-8, whereas FLIP<sub>L</sub> inhibits the second cleavage step of procaspase-8 (25). We investigated whether overexpression of FLIP<sub>L</sub> inhibits FLIP<sub>S</sub> cleavage during treatment with TRAIL and cisplatin. HNSCC-6 cells were infected with Ad/EGFP or Ad/FLAG-FLIP<sub>L</sub> and then treated with TRAIL in combination with cisplatin (Fig. 6). Fig. 6A shows that FLIP<sub>L</sub> (28 kDa) was cleaved to the fragment form of FLIP<sub>S</sub> (23 kDa) during treatment with TRAIL and/or cisplatin (0.5–1 μg/ml) and cisplatin (5–10 μg/ml) in Ad/EGFP-infected HNSCC-6 cells. However, overexpression of FLIP<sub>L</sub> prevented the cleavage of FLIP<sub>S</sub> and PARP and activation of caspase-8 and caspase-3.

**Fig. 3** Synergistic cytotoxicity (A) or enhanced apoptosis (B) by combination treatment with TRAIL and cisplatin in head and neck squamous carcinoma HNSCC-30 cells. A, cells were treated with 0.5 μg/ml TRAIL alone, 10 μg/ml cisplatin alone, or a combination of 0.5 μg/ml TRAIL and 10 μg/ml cisplatin for 24 h, and then survival was analyzed by trypan blue exclusion assay. Data represent two separate experiments. B, cells were treated with TRAIL alone (0.1–1 μg/ml), cisplatin alone (1–10 μg/ml), or a combination of TRAIL (0.1–1 μg/ml) and cisplatin (1–10 μg/ml) for 24 h and then harvested. Lysates from equal amounts of protein (20 μg) were separated by SDS-PAGE and immunoblotted. PARP cleavage and caspase (caspase-8 and caspase-3) activation were analyzed as described in the Fig. 2 legend. Antibody against caspase-9 detects inactive form (48 kDa) and cleaved intermediates (37 kDa). Actin is shown as an internal standard.
We further examined the Ad/FLAG-FLIP\textsubscript{S}-infected HNSCC-6 cells with high dose of cisplatin (20–100 μg/ml). The combined treatment with 1 μg/ml TRAIL and 50–100 μg/ml cisplatin results in the cleavage of endogenous FLIP\textsubscript{S} as well as exogenous FLAG-FLIP\textsubscript{S} in HNSCC-6 cells (Fig. 6B). Fig. 7 shows a schematic diagram of a model based on the current data. This model illustrates that an activated caspase-3 cleaves FLIP\textsubscript{S}. Previous studies have shown that during the execution phase of apoptosis, caspase-3 is responsible for the proteolysis of various substrates, each of which contains a common Asp-Xaa-Xaa-Asp (DXXD) motif (26). Fig. 7 shows a putative cleavage site at Asp\textsuperscript{42}-Ile\textsuperscript{43}.

**DISCUSSION**

In the present study, we observed that the combination of cisplatin and TRAIL resulted in synergistic induction of cell death in HNSCC-6 and HNSCC-30 cells (Figs. 1 and 3). The synergistic cytotoxicity is probably due to the augmentation of caspase-mediated apoptosis (Fig. 2). The enhancement of TRAIL-mediated apoptosis by chemotherapeutic agents has been explained by up-regulation of DR4 and DR5 receptors or down-regulation of FLIP (14–16, 24, 27). However, our data show that intracellular levels of DR4, DR5, DcR2, and FLIP\textsubscript{L} were not changed during treatment with TRAIL in combination with cisplatin.
with cisplatin in HNSCC-6 cells (Fig. 4, A and C). There were no conclusive results when the surface expression of TRAIL receptors was examined during combination treatment with TRAIL and cisplatin (Fig. 4B). This discrepancy may be due to the fact that regulation of DR5 expression by genotoxic drugs is dependent on the presence of wild-type p53 (15, 21, 27, 28), and HNSCC-6 cells contain mutant p53. However, recent reports demonstrate that expression of the DR4 receptor is regulated in a p53-independent manner (29). These results are consistent with previous reports in breast and ovarian cancer cells in which there was no correlation between enhancement in TRAIL-mediated apoptosis by chemotherapeutic agents and DR levels (13, 30).

Previous studies have shown that several FLIP splice variants exist on the mRNA level, but two endogenous forms, FLIP_L and FLIP_S, are detected on the protein level (31, 32). The role of FLIP in apoptosis signaling has been controversially discussed. Some reports have described it as an antiapoptotic molecule (33–35), and others have described it as a proapoptotic molecule (34, 36, 37). Our studies reveal that FLIPS has an antiapoptotic function (Fig. 6). Recent studies show that FLIP_L and FLIP_S prevent caspase-8 activation at different levels of procaspase-8 processing at the DISC (25). FLIP contains DEDs that are similar to the corresponding segment in Fas-associated death domain and caspase-8 (38). FLIPL possesses two DEDs and one caspase-like domain in which tyrosine is substituted for the active cysteine residue necessary for enzymatic activity. FLIP_S possesses two DEDs but no caspase-like domain that is similar to viral FLIP (vFLIPS; Ref. 31). FLIP is recruited to the DISC and inhibits caspase-8 activation. Expressed at high levels in stable transfectants, FLIP completely blocks receptor-ligand apoptotic death through inhibition of caspase-8 processing at the DISC. Interestingly, recent studies revealed that FLIP_S completely inhibits cleavage of procaspase-8, whereas FLIP_L inhibits the second cleavage step of procaspase-8 (25). In this study,
we observed that FLIPₘ, but not FLIPᵢₗ, was cleaved during treatment with 10 μg/ml cisplatin. The cleavage of FLIPₘ was promoted by combined treatment with TRAIL and cisplatin (Fig. 4C). Our data also suggest that caspase-3 is involved in the cleavage of FLIPₘ (Fig. 5). At the present time, we can only speculate how the combination of TRAIL and cisplatin promotes the cleavage of FLIPₘ. Previous studies have shown that chemotherapeutic drugs can activate the initiator caspase-9 by stimulating cytochrome c release from the mitochondria (39, 40). Our previous studies have shown that an increase in cytochrome c release promotes TRAIL-induced apoptosis (18). Overexpression of Bcl-2 blocks the synergistic effect by inhibiting cytochrome c release (18). Thus, one possibility is that combined treatment with TRAIL and cisplatin enhances apoptosis by promoting cytochrome c release. This possibility needs to be examined.

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