Enhancement of Apo2L/TRAIL-mediated cytotoxicity in esophageal cancer cells by cisplatin

Wilson S. Tsai, Wen-Shuz Yeow, Alex Chua, Rishindra M. Reddy, Duc M. Nguyen, David S. Schrump, and Dao M. Nguyen

Section of Thoracic Oncology, Surgery Branch, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, Maryland

Abstract
Although expressing adequate levels of functional tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) receptors DR4/DR5, significant proportion of cancer cells exhibit resistance to the cytotoxic effect of this ligand. Exposure of Apo2L/TRAIL-refractory cancer cells to cytotoxic chemotherapeutic agents enhances their sensitivity to Apo2L/TRAIL cytotoxicity. This study aims to elucidate the molecular mechanism responsible for the cisplatin-mediated enhancement of Apo2L/TRAIL sensitivity in cultured esophageal cancer cells. Exposure of cancer cells to sublethal concentrations of cisplatin resulted in profound potentiation of their susceptibility to Apo2L/TRAIL cytotoxicity as indicated by 2- to >20-fold reduction in Apo2L/TRAIL IC50 values. Significant activation of caspase-8, caspase-9, and caspase-3 was observed only in cells treated with cisplatin/Apo2L/TRAIL combination and not in those exposed to either agent alone. More importantly, activation of these key caspases was significantly abrogated by overexpression of Bcl2 or by the selective caspase-9 inhibitor. This observation strongly suggested that caspase-8 activation in cells treated with the cisplatin/Apo2L/TRAIL combination was secondary to the mitochondria-mediated amplification feedback loop and activation of the executioner caspase-3 was dependent on the recruitment of the intrinsic pathway characteristic of the type II cell. Profound combination-mediated cytotoxicity and induction of apoptosis was completely suppressed either by Bcl2 overexpression or by inhibition of caspase-9 activity, which conclusively pointed to the essential role of the mitochondria-dependent death signaling cascade in this process. Cisplatin sensitizes esophageal cancer cells to Apo2L/TRAIL cytotoxicity by potentiation of the mitochondria-dependent death signaling pathway that leads to amplification of caspase activation, particularly caspase-8, by the feedback loop to efficiently induce apoptosis. [Mol Cancer Ther 2006;5(12):2977–90]

Introduction
Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL), the apoptosis-inducing member of the tumor necrosis factor superfamily, has been critically evaluated as a novel biological agent for cancer therapy (1–5). In contrast to tumor necrosis factor, which targets tumor microvasculature to induce thrombosis and tumor necrosis as the principal mechanism for its anticancer property (6), TRAIL exerts its tumoricidal effect by directly inducing apoptosis of susceptible cancer cells via a receptor-mediated process (7–10). More importantly, TRAIL, in its Zn2+-containing homotrimeric form known as Apo2L/TRAIL (11), is tumor selective (12) in that it rapidly and profoundly induces apoptosis of susceptible cancer cells while sparing normal cells especially primary human hepatocytes. Systemic administrations in nonhuman primates were well tolerated with no apparent toxicity to the liver or the bone marrow (4).

The receptor repertoire of TRAIL is complex and consists of five members: two functional death receptors (DR4 and DR5) that contain a conserved intracellular death domain motif; two decoy receptors (DcR1 and DcR2) that can bind TRAIL but lack the functional intracellular death domain, thus incapable of transducing a death signal; and one soluble protein (osteoprotegerin) that binds TRAIL but at a very low affinity at physiologic condition (1). The molecular events downstream of DR4/DR5 activation by TRAIL are well described (1, 9, 13). Binding of trimerized TRAIL to its cognate functional receptors DR4 and/or DR5 results in receptor aggregation and recruitment of FADD and procaspase-8/procaspase-10 to the intracellular domain of receptors to form the death-inducing signaling complex (DISC) that process these zymogens to produce proteolytically active caspase-8/caspase-10. The DISC-mediated activation of the physiologically important caspase-8 then can cleave and activate caspase-3, caspase-6, and caspase-7, leading to apoptosis via a signaling cascade known as the extrinsic pathway, in contrast to the mitochondria-mediated intrinsic death signaling pathway activated by cytotoxic stresses (14, 15). Moreover, activated caspase-8 can also process Bid to yield truncated Bid (16, 17). Truncated Bid translocates to the mitochondria and, via interaction with Bak and Bax (16–18) on the mitochondria, mediates the release of multiple proapoptotic proteins, including...
cytochrome c, apoptosis-inducing factor, and SMAC/DIABLO, thus linking the extrinsic to the intrinsic death signaling pathways (14, 15, 19, 20). Cytochrome c, together with cytosolic apoptotic protease-activating factor-1, procaspase-9, and ATP, forms the apoptosome that activates caspase-9. Activated caspase-9 processes caspase-3 (and caspase-7) and the former of which can proteolytically cleave and activate caspase-8, thus forming a positive amplification feedback loop to further activate the apical caspase-8 (21). Death ligand-mediated induction of apoptosis is further classified into type I or type II depending on the involvement of the intrinsic (mitochondria mediated) death signaling cascade in the effective execution of apoptosis. Type I cells undergo death receptor-mediated apoptosis independent of mitochondria and are thus not sensitive to Bcl2/BclXL, whereas type II cells rely on the intrinsic pathway for efficient apoptosis, and apoptosis is abrogated by Bcl2, by BclXL, or by the selective caspase-9 inhibitor (z-LEHD-fmk; ref. 22).

Although the majority of cultured cancer cells of different histology show a certain degree of sensitivity to the cytotoxic effect of TRAIL (4), it has been well documented that pretreating these cells with genotoxic agents, such as cisplatin [cis-diaminedichloroplatinum (CDDP)], or irradiation significantly enhances the sensitivity of cancer cells to this apoptosis-inducing ligand. The molecular basis of such enhancement effect is complex, cell dependent, and not completely elucidated (2, 13, 23–27). Up-regulation of receptor expression, up-regulation of DISC-mediated signaling, alteration of intracellular signal transduction, and down-regulation of natural inhibitor proteins of the caspase cascade following treatment of cancer cells with sensitizers have been postulated as the basis of TRAIL sensitization. In this study, we examined the molecular basis of CDDP-mediated enhancement of cellular responsiveness to Apo2L/TRAIL in esophageal cancer cells by examining the dynamics of caspase activation in cancer cells treated with Apo2L/TRAIL with or without CDDP pretreatment and the ability of Bcl2 or the selective caspase-9 inhibitor to interfere with this process to explore the role of the intrinsic death signaling cascade in this process.

Materials and Methods

Cells and Reagents

Esophageal cancer cell lines SKGT5, TE2, TE3, and TE12 were grown in RPMI 1640 culture supplemented with glutamine (1 mol/L), penicillin (100 units/mL)/streptomycin (100 μg/mL), and 10% (v/v) FCS. Primary normal human dermal fibroblasts, skin keratinocytes, bronchial epithelia, human umbilical vascular endothelial cells, and their specific culture media were purchased from Clonetics Corp. (Walkersville, MD). Apo2L/TRAIL was provided by Genentech (South San Francisco, CA) under a collaborative research and development agreement, aliquoted, and stored in –80°C until thawed before each use. Cisplatin was purchased from the pharmacy of the Clinical Center (NIH, Bethesda, MD). General caspase inhibitor z-VAD-fmk, caspase-9 selective inhibitor z-LEHD-fmk, and colorimetric caspase-8, caspase-9, and caspase-3 activity assay kits were purchased from R&D Systems (Minneapolis, MN). Bcl2-overexpressing stable transfectants of TE2 (TE2Bcl2) and TE12 (TE12Bcl2) cells were created by retrovirally mediated gene transfer using Bcl2-expressing viral vector containing green fluorescent protein as a selectable marker (generously provided by P. Robbins, Surgery Branch, National Cancer Institute, NIH) and previously published transfection techniques (28). Vector control stable transfectants of similar cancer cells were created using green fluorescent protein–expressing retrovirus. The magnitude of green fluorescent protein fluorescence in Bcl2 stable transfectants closely correlated with the level of Bcl2 expression (28). Cells with the highest level of green fluorescent protein were selected by cell sorting for further culture and expansion. Bcl2 was detected using intracellular staining with phycoerythrin (PE)-conjugated anti-Bcl2 monoclonal antibody (clone Bcl2/100, Alexis Biochemicals, San Diego, CA) after cell fixation and permeabilization using the Cytofix/Cytoperm kit from BD Biosciences (San Jose, CA). Flow cytometry confirmed 100% of cells expressing high levels of Bcl2. Western blot analysis showed very high levels of Bcl2 in all Bcl2-expressing stable transfectants.

Flow Cytometric Analysis of TRAIL Receptors

Cancer cells (controls or treated with CDDP at 1.0 or 2.0 μg/mL for 24 h) were washed with PBS with 1% bovine serum albumin and incubated with biotinylated mouse anti-human antibodies for DR4, DR5, DcR1, and DcR2 (1.0 μg/500,000 cells; R&D Systems) for 30 min at room temperature. Excess unbound antibodies were removed by one wash with PBS and 5% bovine serum albumin, and cells were further incubated with streptavidin-PE (50 μL/500,000 cells; R&D Systems) for 30 min and washed once before submission for flow cytometry. The levels of receptor expression were quantified by the PE mean fluorescence intensity index (ratio of PE mean fluorescence intensity of cells incubated with antireceptor antibodies and the background PE mean fluorescence intensity in cells incubated with IgG isotype control) and the percentages of cells gated positive for receptor expression.

In vitro Cytotoxicity and Apoptosis Assays

Cells were seeded in 96-well microtiter plates (15,000 per well). After an overnight incubation, they were treated with CDDP (0.5, 1.0, and 2.0 μg/mL) for 24 h, and following removal of drug-containing medium, they were either further incubated with fresh medium alone or exposed to Apo2L/TRAIL (5–100 ng/mL) for 36 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cell viability after Apo2L/TRAIL or CDDP/Apo2L/TRAIL sequential combination treatments was expressed as percentages of cell viability of appropriate controls (cells grown in normal medium as controls for Apo2L/TRAIL-treated cells and cells grown in CDDP-containing medium as controls for those treated with the combinations to normalize for the
mild growth-inhibitory effect of CDDP pretreatment). Cytotoxicity dose-response curves were constructed for cells treated with Apo2L/TRAIL alone or in combination with CDDP, and Apo2L/TRAIL IC_{50} values (concentrations of the ligand that inhibit 50\% of cell viability with or without CDDP pretreatment) were derived from respective curves. Apoptosis in cells treated with Apo2L/TRAIL, CDDP, or CDDP/Apo2L/TRAIL was determined by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based ApoBrdU assay or by staining with PE-conjugated Annexin V (BD PharMingen, San Diego, CA) and flow cytometry.

**Caspase Activity Assays**

Specific proteolytic activity of the caspase-3, caspase-8, and caspase-9 in cells treated with Apo2L/TRAIL, CDDP, or CDDP/Apo2L/TRAIL combination was determined by the colorimetric assay kits. Cells were seeded (8 x 10^5 per well) in six-well plates and subsequently treated with CDDP (1.0 \mu g/mL for 24 h), Apo2L/TRAIL (50 or 100 ng/mL), or CDDP/Apo2L/TRAIL. Cells incubated with complete medium served as baseline controls. Cells were harvested at intervals after exposure to Apo2L/TRAIL. Selective caspase activity in cell lysates was measured and expressed as fold increase from baseline controls. In caspase inhibition experiments, z-VAD-fmk (60 \mu mol/L) or z-LEHD-fmk (40 \mu mol/L) was present in the cultured medium for the entire duration of drug treatments.

**Western Blots**

Following treatments as per indicated in individual figure legends, whole-cell extracts were prepared in cell lysis buffer (Cell Signaling Technology, Beverly, MA) supplemented with 1 mmol/L phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Cell extracts (50 \mu g) were separated on a gradient 4\% to 20\% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with primary antibodies against FLIP, Bid, Bax, Bak, BclXL, XIAP (Cell Signaling Technology, Danvers, MA), caspase-8, caspase-3, McI1 (BD Biosciences), Bcl2 (Upstate USA, Inc., Charlottesville, VA), cIAP-1 (R&D Systems), and \beta-actin (EMD Biosciences, La Jolla, CA), which were diluted at 1:1,000 with 3\% nonfat dry milk. The blots were developed using a peroxidase-conjugated secondary antibody and visualized with SuperSignal West Dura (Pierce Biotechnology, Rockford, IL).

**Statistical Analysis**

Supra-additive apoptosis, defined as the apoptosis induced by the drug combinations, is, by statistical analysis, significantly greater than the algebraic sum of apoptosis induced by individual drug treatment. Data are expressed as mean ± SE. Two-tailed Student’s t test was used for statistical analysis, and Ps < 0.05 were considered statistically significant.

**Results**

**Sensitivity of Esophageal Cancer Cells to Apo2L/TRAIL**

Indirect immunofluorescence staining and flow cytometric analysis for DR4 and DR5 expression indicated that >80\% of esophageal cancer cells were positively stained for either DR4 or DR5, with the mean fluorescence intensity indices ranging from 3 to 12, whereas the expression levels of DcR1 and DcR2 were more variable between cell lines (data not shown; Fig. 1). Initial experiments were done to assess the intrinsic sensitivity of esophageal cancer cells to Apo2L/TRAIL. Continuous exposure of esophageal cancer cells grown in 10\% FCS/RPMI 1640 to this ligand for 36 h resulted in a clear dose-dependent reduction of cell viability only in one of the four cell lines evaluated. Apo2L/TRAIL mediated a mild growth-inhibitory effect in TE12 cells with an estimated IC_{50} value of 180 ± 12 ng/mL. On the other hand, DR4/DR5-positive SKGT5, TE2, and TE3

![Figure 1](https://example.com/image1.png)

**Figure 1.** Flow cytometric analysis of DR4 and DR5 expression on cultured esophageal cancer cell lines. All esophageal cancer cells express high levels of DR4/DR5, whereas the level of DcR1 and DcR2 is more variable between cell lines (data not shown).
cells were totally refractory to the cytotoxic effect of this ligand (IC50 values, >500 ng/mL; Fig. 2A). Apoptosis, readily detectable in sensitive TE12 cells following 24-h treatment with high concentrations of Apo2L/TRAIL (100 or 200 ng/mL), was completely abrogated by overexpression of Bcl2. This indicated TE12 cells as type II cells (Fig. 2B). Low percentages of Annexin V–positive cells noted in TE2 cells similarly treated with high concentrations of Apo2L/TRAIL were only partially suppressed by Bcl2, raising the possibility that both type I and type II pathways are operational in this cell line. Pretreatment both Apo2L/TRAIL-sensitive and Apo2L/TRAIL-resistant esophageal cancer cells with sublethal concentrations of CDDP (0.5–2.0 μg/mL) for 24 h resulted in significant enhancement of Apo2L/TRAIL-mediated cytotoxicity (Fig. 3A). At these concentrations, CDDP mediated mild cycle arrest but minimal apoptosis resulting in <30% reduction of cell viability compared with untreated control cells by MTT assay. The CDDP-mediated sensitization of esophageal cancer cells to Apo2L/TRAIL was most easily appreciated when the growth-inhibitory effect of the CDDP/Apo2L/TRAIL combinations was normalized for the mild CDDP-induced cytotoxicity as presented in the respective dose-response curves shown in Fig. 3A. CDDP pretreatment drastically reduced the Apo2L/TRAIL IC50 values of esophageal cancer cells. For instance, the baseline IC50 of TE12 cells was 180 ± 12 ng/mL compared with 50 ± 4 ng/mL in CDDP-treated cells (1.0 μg/mL) or 22 ± 3 ng/mL in CDDP-treated cells (2.0 μg/mL); baseline IC50 value of the Apo2L/TRAIL-resistant TE2 cells was >1,000 ng/mL compared with 85 ± 6 ng/mL in CDDP-treated cells (1.0 μg/mL) or 12 ± 2 ng/mL in CDDP-treated cells (2.0 μg/mL; P < 0.001, by ANOVA and pairwise analysis with Bonferroni test between CDDP-treated cells and untreated controls; n = 4). More importantly, although strongly cytotoxic to cancer cells, this combination had no effect on normal cells, primary cultured human umbilical vascular endothelial cells, primary human skin fibroblasts, or freshly isolated peripheral blood mononuclear cells (Fig. 3B). Moreover, profound induction of apoptosis was observed in esophageal cancer cells treated with the sequential CDDP/Apo2L/TRAIL in complete concordance with the enhanced cytotoxicity mediated by this drug combination as assessed by the MTT assays shown in Fig. 3A. Although neither CDDP nor low concentrations of Apo2L/TRAIL alone induced any cell death, 60% to 85% of cancer cells treated with the CDDP/Apo2L/TRAIL combinations underwent apoptosis as determined by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based ApoBrdU assay and flow cytometry (Fig. 4A and B). The combination-induced apoptosis was clearly supra-additive at two different concentrations of CDDP (0.25 and 0.50 μg/mL for SKGT5 and 1.0 and 2.0 μg/mL for TE2 and TE12 cells) and of Apo2L/TRAIL (25 or 50 ng/mL).

The Effect of CDDP on the Expression of DISC Components and Members of the Bcl2 Superfamily

To elucidate the putative molecular mechanism(s) responsible for the synergistic cytotoxic effect of this CDDP/Apo2L/TRAIL combination, we first examined the effect of CDDP on the phenotypic expression of components of the apoptosis-inducing pathways (i.e., the DISC and the mitochondria-associated members of the Bcl2 superfamily). Cisplatin treatment, in contrast to previously reported findings in other cultured cancer cells (27, 29), did not significantly alter the phenotypic expression of the DISC and the mitochondria-associated components and Members of the Bcl2 Superfamily.

Figure 2. A, intrinsic sensitivity of esophageal cancer cells to Apo2L/TRAIL. Cells (2.0 × 104 per well) were continuously treated with varying concentrations of Apo2L/TRAIL (5–100 ng/mL) for 36 h. Cell viability was determined by MTT assay. Points, mean of four independent experiments; bars, SE. B, Apo2L/TRAIL-mediated induction of apoptosis in TE2 and TE12 cells was blocked by overexpression of Bcl2. Columns, mean of three independent experiments; bars, SE.

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treated with either drug alone. There was no discernible alteration of the levels of Bax, Bak, Bcl2, BclXL, McI, and IAPs in treated cells. As Apo2L/TRAIL, at high concentrations, mediates apoptosis via the type II pathway (Fig. 3A), we focused our investigation on evaluating factors that regulate activation of key caspases in the apoptosis-inducing cascade (caspase-8, the apical caspase of the extrinsic pathway; caspase-9, the initiator caspase of

![Graphs showing cytotoxicity enhancement](image)

**Figure 3.** A, significant enhancement of cytotoxicity by the CDDP + Apo2L/TRAIL combination in cultured esophageal cancer cells. Pretreating esophageal cancer cells, either intrinsically sensitive or resistant to Apo2L/TRAIL, with CDDP (0.5 – 2.0 μg/mL for TE2, TE3, and TE12 or 0.25 – 1.0 μg/mL for SKGTS) resulted in profound dose-dependent enhancement of cytotoxicity. Points, mean of four independent experiments; bars, SE. B, no cytotoxicity was observed in normal cells (normal human bronchial epithelial cells (NHBE), peripheral blood mononuclear cells (PBMC), dermal fibroblasts, or human umbilical vascular endothelial cell (HUVEC)) treated with either Apo2L/TRAIL or CDDP/Apo2L/TRAIL combination. Points, mean of four independent experiments; bars, SE.
the intrinsic pathway; and caspase-3, the executioner caspase of both pathways) in cells treated with CDDP, Apo2L/TRAIL, or the drug combinations as well as the functional significance of the mitochondria-derived caspase-9 in mediating apoptosis following combination treatment.

**Functional Activation of Caspase-8, Caspase-9, and Caspase-3 following CDDP + Apo2L/TRAIL Treatment**

Cultured esophageal cancer cells continuously treated with Apo2L/TRAIL (50 ng/mL) with or without CDDP pretreatment (2.0 μg/mL for 24 h) were harvested at 2-h intervals immediately following the onset of exposure to the ligand and assayed for the enzymatic activity of caspase-8, caspase-9, and caspase-3. Untreated or CDDP-pretreated cells served as respective controls. There was no detectable caspase activation following CDDP treatment in all cell lines. Exposure to Apo2L/TRAIL (50 ng/mL) alone resulted in detectable activation of these caspases only in the Apo2L/TRAIL-sensitive TE12 cells and not in the Apo2L/TRAIL-resistant TE2 cells (Fig. 7).

Remarkably, the CDDP/Apo2L/TRAIL combination induced supra-additive activation of caspase-8, caspase-9, and caspase-3 in both Apo2L/TRAIL-sensitive TE12 and Apo2L/TRAIL-resistant TE2 cells. Functionally, elevated caspase-8 activity following combination treatment was associated with cleavage of its substrate BID, providing a link between extrinsic and intrinsic caspase cascades (Fig. 7). The functional assay of caspase activity mirror imaged the Western blot analysis, indicating very high levels of mature activated caspase-8 and caspase-3 (Fig. 6) at similar time points. In the absence of phenotypic upregulation of components of the DISC in CDDP-treated cells, one had to wonder by which mechanism CDDP/Apo2L/TRAIL-treated cells achieved such a high level of

![Figure 4](image_url)

**Figure 4.** Supra-additive induction of apoptosis by the CDDP/Apo2L/TRAIL combinations in esophageal cancer cells at two different concentrations of CDDP (A) or Apo2L/TRAIL (B) as indicated. Apoptosis of cancer cells 48 h after the onset of Apo2L/TRAIL exposure was quantitated by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)–based ApoBrdU assay. At each experimental condition, percentages of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive cells following combination treatments are consistently and significantly higher than the algebraic sum of percentages of apoptosis induced by either CDDP alone or Apo2L/TRAIL alone. Columns, mean (n = 3); bars, SE. #, P < 0.001; *, P < 0.001; +, P < 0.001.

![Figure 5](image_url)

**Figure 5.** A, treating TE2, TE3, and TE12 esophageal cancer cells with CDDP (1.0 μg/mL) for 24 h did not change the levels of receptor expression in TE esophageal cancer cells. *, P = 0.28, two-tailed Student’s t test. Similar observation was made following treatment with 2.0 μg/mL CDDP. Columns, mean of the mean fluorescence intensity index of three independent experiments; bars, SE. B, Western blot analysis of DISC-related proteins and proapoptotic/antiapoptotic proteins of the Bcl2 family in TE cells treated with CDDP (1.0, 2.0, or 4.0 μg/mL) for 24 h. Similar findings were obtained in cells harvested at 20 or 28 h after the onset of CDDP treatment (data not shown).
caspase-8 activation. One possibility is that it is due to an increase of DISC assembly and function, whereas the other is that it is amplified by the mitochondria-dependent feedback loop. To elucidate further the position of activated caspase-8 in the hierarchy of the caspase cascade in combination-treated cells, we assayed for the activity of caspase-8, caspase-9, and caspase-3 in cells treated with CDDP alone, Apo2L/TRAIL (50 ng/mL for TE12 and 100 ng/mL for TE2 cells) alone, or the drug combinations with or without the selective caspase-9 inhibitor (40 μmol/L; Fig. 8). Supra-additive activation of caspase-9 and the downstream executioner caspase-3 in cells treated with drug combination, as expected, was totally suppressed by the selective caspase inhibitor. It is, however, interesting to observe that the selective caspase-9 inhibitor completely abrogated the elevated caspase-8 activity in cancer cells exposed to the drug combination, providing evidence that caspase-8 activation in this setting was mediated by the mitochondria-derived caspase-9 via the amplification feedback loop and probably not derived from functional up-regulation of DISC activity. Such profound caspase activation was most likely responsible for the cytotoxic effect of this combination in cultured esophageal cancer cells. To define the functional significance and the correlation between caspase activation and cytotoxicity mediated by this combination, we used z-VAD-fmk or z-LEHD-fmk to suppress either total or caspase-9 activity in these esophageal cancer cells.

**CDDP/Apo2L/TRAIl-Mediated Cytotoxicity and Apoptosis Is Caspase-9 Dependent or Bcl2 Sensitive**

CDDP + Apo2L/TRAIl-mediated cytotoxicity in TE2 and TE12 was significantly abrogated not only by z-VAD-fmk (60 μmol/L) but also by z-LEHD-fmk (40 μmol/L; Fig. 9A), indicative that this process was caspase mediated and strongly dependent on the mitochondria-regulated caspase-9 (type II pathway). Moreover, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–based ApoBrdU assay indicated that the selective caspase-9 inhibitor z-LEHD-fmk alone was sufficient in blocking the profound apoptosis induced by the drug combination in TE2, TE3, and TE12 cells (Fig. 9B). As caspase-9 is the initiator caspase of the intrinsic mitochondria-dependent
caspase activation cascade, apoptosis induced by this drug combination was therefore most likely mediated by caspase activation via this pathway. To definitely examine the role of the mitochondria in this process, we created stable transfectants of TE2 and TE12 cells that expressed high levels of the antiapoptotic protein Bcl2 using a retroviral vector. Similar to the findings that caspase-9 inhibitor blocked caspase-8 activation following Apo2L/TRAIL or Apo2L/TRAIL + CDDP treatment, Bcl2 overexpression completely prevented not only the increased caspase-9 and caspase-3 activity but also the robust caspase-8 activation following similar treatments (Fig. 10A). This observation conclusively confirmed the view that profound elevation of caspase-8 activity following CDDP + Apo2L/TRAIL exposure was the result of the mitochondria-mediated caspase-8 amplification feedback loop. As expected, Bcl2-overexpressing TE2 and TE12 cells were significantly resistant to the cytotoxic effect of CDDP + Apo2L/TRAIL (Fig. 10B). Moreover, the reduction of cell viability following Apo2L/TRAIL treatment of the ligand-sensitive TE12 cells was also abrogated by Bcl2 (as also by the selective caspase-9 inhibitor as mentioned above and shown in Fig. 8A), similar to earlier apoptosis data shown in Fig. 2B, affirming the notion that TE12 cells were type II cells. These observations underscore the fact that Apo2L/TRAIL, alone or in combination with CDDP pretreatment, mediates its cytotoxic effect in TE2 and TE12 cells via the Bcl2-dependent mitochondria-regulated intrinsic death signaling cascade.

Figure 7. Time course of caspase activation in TE2 (A) or TE12 (B) cells treated with CDDP + Apo2L/TRAIL combination, although either CDDP alone or Apo2L/TRAIL (50 ng/mL) alone did not affect the level of caspase activity compared with untreated control cells, except mild caspase activation following Apo2L/TRAIL treatment in the ligand-sensitive TE12 cells. Significantly elevated caspase activity was only observed in cells treated with drug combination. Significant reduction of BID was observed in Apo2L/TRAIL (TE12 cells) and in CDDP/Apo2L/TRAIL-treated cells (TE2 and TE12) 12 h after exposure to Apo2L/TRAIL. Elevated levels of caspase-8 activity were correlated with cleavage of BID. Points, mean of four independent experiments; bars, SE.

Discussion

The worldwide incidence of cancers of the esophagus has steadily been increasing in the last 20 years (30, 31). In Western countries, esophageal cancer is frequently diagnosed late in its course for which potential curative therapeutic strategies consisted of surgery, platinum-based chemotherapy, and radiotherapy are often ineffective and carry high incidence of morbidities (30). Moreover, systemic recurrence is common after curative-intent treatments. Adjutant therapy with cytotoxic agents after complete surgical resection of esophageal cancer is not effective and is poorly tolerated because of untoward side effects (30). Better understanding of the molecular basis of carcinogenesis and elucidation of signal transduction pathways regulating cell growth and death in normal cells and their roles in the process of malignant transformation provide the impetus for the development of novel molecularly targeted anticancer therapy. Within this context, therapeutic strategies aiming at direct induction of cell death by activation of the TRAIL receptor-mediated signal transduction pathways have attracted a great deal of attention and, in fact, recently entered early-phase clinical development. Recombinant protein, such as Apo2L/TRAIL (4), or recombinant human agonistic anti-DR4 or anti-DR5 monoclonal antibodies (32) are commonly used to activate TRAIL receptor for the induction of apoptosis of cancer cells. It became clear to investigators in this field that a significant proportion of cultured malignant cells, although expressing the functional TRAIL receptors DR4/DR5, are refractory to the cytotoxic effect of recombinant soluble TRAIL (2). The molecular basis of this intrinsic or acquired resistance to TRAIL-induced cytotoxicity in various cancer cell lines is complex and multifactorial (2, 33). Fortunately, this limitation can be overcome by combining recombinant TRAIL receptor ligands with cytotoxic agents [either standard cytotoxic drugs, such as cisplatin (5, 26, 29, 34), CTP-11 (4), or others (5, 25, 26, 35, 36), or clinically relevant experimental anticancer agents, such as histone deacetylase inhibitors (37–39)]. Although the underlying mechanisms responsible for the synergistic interactions between chemotherapeutics and TRAIL receptor agonists to mediate
profound induction of apoptosis are incompletely understood, it seems that recruitment/activation of the intrinsic death pathway (mitochondria mediated) in combination-treated cells plays the crucial role.

Apo2L/TRAIL-sensitive cells were arbitrarily defined in this study as those with IC50 values of <200 ng/mL following in vitro treatment conditions described in Materials and Methods. Pretreating TE esophageal cancer cells, whether intrinsically sensitive to Apo2L/TRAIL or not, with sublethal concentrations of CDDP profoundly enhanced their susceptibility to this death ligand. The cultured esophageal cancer cells treated with this drug combination behaved as type II cells because the treatment-mediated cytotoxicity was abrogated either by Bcl2 overexpression or by the selective caspase-9 inhibitor (Figs. 2B and 9A). Previous studies have indicated that cytotoxic chemotherapeutic drugs sensitize cultured cancer cells to TRAIL by up-regulation of the receptors DR4/DR5 (25, 27, 29, 40), enhanced DISC formation/function (26, 41, 42), or alterations of the phenotypic expression of proapoptotic/antiapoptotic proteins (25, 34, 43, 44) that is paralleled with an overall increase of caspase-8 activation and massive cell death. Our data did not indicate a significant alteration of TRAIL receptor expression in CDDP-treated cells. The alteration of members of the Bcl2 superfamily (increase of Bak and/or Bax and unchanged levels of Bcl2 and/or BclXL) that was similar to some of the published reports following CDDP treatment hinted the possible functional involvement of the mitochondria pathway. Consistent with previous observations, significant activation of key caspase-8, caspase-9, and caspase-3 was observed in combination-treated cells. Processing of caspase-3 can be mediated by caspase-8 (extrinsic pathway) and/or by caspase-9 (intrinsic pathway). The strong caspase-8 activation in combination-treated TE2 or TE12 cells may be the result of increased DISC activity or secondary to activation by downstream caspases, such as caspase-9, via caspase-3. Detailed analysis of the hierarchical order of caspase activation following cytochrome release and caspase-9 activation has indicated that caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, and caspase-10 are substrates of caspase-9 and caspase-3 is required for the processing of caspase-2, caspase-6, caspase-8, and caspase-10 in this cascade (21). Blocking of caspase-3 activity using a selective inhibitor would abrogate caspase-8 activation if this was indeed secondary to a downstream caspase-dependent feedback loop, but caspase-3 blockade would not discriminate which pathway downstream from the DISC (intrinsic versus extrinsic) is responsible for perpetuation of caspase-8 activation. On the other hand, inhibition of caspase-9 using the selective inhibitor z-LEHD-fmk would block both caspase-3 and caspase-8 activation (in addition to inhibiting caspase-9 activity) if caspase-9 was predominantly responsible for caspase-3 activation and if proteolytic processing of caspase-8 was secondary to caspase-3 activation by the mitochondria-mediated intrinsic pathway. This was exactly what we observed in TE esophageal cancer cells treated with the CDDP/Apo2L/TRAIL combinations (Fig. 8). Thus, the high level of caspase-8 activity was attributable to the amplification feedback loop mediated by mitochondria-derived caspase-9 and caspase-3 activation. DISC-mediated activation of caspase-8 was initially thought to be due to autocalytic cleavage.
Figure 9. A, almost complete abrogation of CDDP + Apo2L/TRAIL cytotoxicity by z-VAD-fmk or z-LEHD-fmk in TE2 and TE12 cells. Cell viability following treatments with or without caspase inhibitors was determined by MTT and expressed as percentages of cell viability of untreated controls. Columns, mean of four independent experiments; bars, SE. #, $P < 0.01$, between normal medium and Z-VAD-treated or caspase-9 inhibitor-treated cells; *, $P < 0.01$, between normal medium and Z-VAD-treated or caspase-9 inhibitor-treated cells. B, CDDP + Apo2L/TRAIL-mediated apoptosis was completely blocked by z-LEHD-fmk. Representative data of three independent experiments with similar results.
Figure 10. A, inhibition of caspase activation by Bcl2 overexpression. Parental control, vector control, or Bcl2 stable transfectants were treated with CDDP, Apo2L/TRAIL, or CDDP + Apo2L/TRAIL combination, and the caspase activity at 8 h after Apo2L/TRAIL treatment was determined by the colorimetric assay. In addition to inhibition to suppressing caspase-9 activity, Bcl2 significantly inhibited the activation of caspase-8 and caspase-3. Columns, mean of four independent experiments; bars, SD. B, abrogation of CDDP + Apo2L/TRAIL-mediated cytotoxicity as evaluated by the MTT cell viability assay in TE2 and TE12 cell lines overexpressing Bcl2. Columns, mean of four independent experiments; bars, SD. *, $P < 0.01$, between parental cell, vector control cells, and Bcl2-overexpressing cells; #, $P < 0.01$, between parental cell, vector control cells, and Bcl2-overexpressing cells.
of procaspase-8 recruited to the DISC to yield activated caspase-8 (45), but more recently, homodimerization, but not proteolytic cleavage, is responsible for this mode of caspase-8 activation following death receptor activation (46, 47). On the other hand, activation of cytosolic procaspase-8 can alternatively occur by activated caspase-3-mediated or caspase-9-mediated proteolytic cleavage via the intrinsic death signaling subsequent to death receptor activation (21) or independent of the formation of DISC as observed in cancer cells exposed to cytotoxic chemotherapeutic drugs (48). Careful analysis of the results of the caspase activity assay indicated that Apo2L/TRAIL at high concentrations (100 ng/mL for TE2 and 50 ng/mL for TE12 cells) alone increased caspase-8 proteolytic activity in TE2 (2-fold of control) and TE12 (3-fold of control; Fig. 7A and B) and induced low levels of cytotoxicity and apoptosis (Figs. 2B and 9A). The ligand-mediated activation of caspase-8, caspase-9, and caspase-3 and accompanying cytotoxicity was clearly suppressed either by the caspase-9 inhibitor z-LEHD-fmk or by overexpression of Bcl2, suggesting that activation of key caspases and induction of apoptosis in TE2 and TE12 cell lines by high concentrations of Apo2L/TRAIL were the results of the mitochondria-dependent amplification loop characteristic of the type II cells. In this regard, our findings were in complete agreement with previous report describing the involvement of the mitochondria pathway-mediated amplification loop (25). In ligand-sensitive TE12 cells, the small amount of caspase-8 activity (1.2- to 1.5-fold of control baseline activity) following Apo2L/TRAIL treatment in Bcl2 cells or in caspase-9 inhibitor-treated controls may indicate the low levels of DISC-derived activation of caspase-8 revealed by abrogation of the intrinsic pathway cascade and its caspase amplification effect. Another possibility was that this represented residual caspase-8 activity not completely abrogated by z-LEHD-fmk or Bcl2. As such, the initial DISC-activated caspase-8 activation required for “jump starting” of the amplification feedback loop may be too minute to be detected by the weakly sensitive colorimetric caspase assay or it occurred within minute of ligand/receptor engagement and was totally missed by the coarse time course experiments done in this study (earliest time point was 2 h after addition of Apo2L/TRAIL to the culture medium). It seems that CDDP synergistically interacted with Apo2L/TRAIL to induce massive apoptosis via further activation of the mitochondria-dependent pathway in type II esophageal cancer cells.

The balance of proapoptotic and antiapoptotic members of the Bcl2 superfamily within the cells serves as a rheostat for the induction of apoptosis following exposure to cytotoxic stress (49). Cisplatin altered the levels of Bax/Bak and Bcl2/BclXL in TE esophageal cancer cells. This alteration of the ratio of proapoptotic versus antiapoptotic proteins in CDDP-treated cells probably favored a proapoptotic cellular milieu and possibly making the mitochondria more susceptible to a second apoptosis signal provided by the subsequent Apo2L/TRAIL exposure. This in a sense serves to prime the mitochondria to a second proapoptotic signal—in this case, activation of caspase-8 and generation of truncated BID by Apo2L/TRAIL. Other studies showed the involvement of the mitochondria pathway in chemotherapeutic drugs (48). Careful analysis of the results of the caspase activity assay indicated that Apo2L/TRAIL at high concentrations (100 ng/mL for TE2 and 50 ng/mL for TE12 cells) alone increased caspase-8 proteolytic activity in TE2 (2-fold of control) and TE12 (3-fold of control; Fig. 7A and B) and induced low levels of cytotoxicity and apoptosis (Figs. 2B and 9A). The ligand-mediated activation of caspase-8, caspase-9, and caspase-3 and accompanying cytotoxicity was clearly suppressed either by the caspase-9 inhibitor z-LEHD-fmk or by overexpression of Bcl2, suggesting that activation of key caspases and induction of apoptosis in TE2 and TE12 cell lines by high concentrations of Apo2L/TRAIL were the results of the mitochondria-dependent amplification loop characteristic of the type II cells. In this regard, our findings were in complete agreement with previous report describing the involvement of the mitochondria pathway-mediated amplification loop (25). In ligand-sensitive TE12 cells, the small amount of caspase-8 activity (1.2- to 1.5-fold of control baseline activity) following Apo2L/TRAIL treatment in Bcl2 cells or in caspase-9 inhibitor-treated controls may indicate the low levels of DISC-derived activation of caspase-8 revealed by abrogation of the intrinsic pathway cascade and its caspase amplification effect. Another possibility was that this represented residual caspase-8 activity not completely abrogated by z-LEHD-fmk or Bcl2. As such, the initial DISC-activated caspase-8 activation required for “jump starting” of the amplification feedback loop may be too minute to be detected by the weakly sensitive colorimetric caspase assay or it occurred within minute of ligand/receptor engagement and was totally missed by the coarse time course experiments done in this study (earliest time point was 2 h after addition of Apo2L/TRAIL to the culture medium). It seems that CDDP synergistically interacted with Apo2L/TRAIL to induce massive apoptosis via further activation of the mitochondria-dependent pathway in type II esophageal cancer cells.

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References


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