Camptothecin- and etoposide-induced apoptosis in human leukemia cells is independent of cell death receptor-3 and -4 aggregation but accelerates tumor necrosis factor–related apoptosis-inducing ligand–mediated cell death

Stephane Bergeron,1 Myriam Beauchemin,1 and Richard Bertrand1,2

1Centre de recherche, Centre hospitalier de l’Université de Montréal–Hôpital Notre-Dame and Institut du cancer de Montréal and 2Département de médecine, Université de Montréal, Montréal, Quebec, Canada

Abstract
During camptothecin- and etoposide (VP-16)-induced apoptosis in HL-60 cells, the expression level of cell death receptor-3 (DR3), cell death receptor-4 (DR4), and FAS remained mostly unchanged, whereas the expression of silencers of death domain (SODD) and FLICE inhibitory proteins, inhibitors of the cell death receptor signaling pathways, decreased substantially. By indirect immunofluorescence and immunoperoxidase imaging and with gel filtration column chromatography, we observed rapid aggregation at the cell surface and the appearance of high molecular weight protein complexes primarily involving DR3, and DR3 and DR4 after camptothecin and VP-16 treatment, respectively. Both drugs failed to rapidly promote FAS aggregation in these cells. The high expression level of SODD or of dominant negative forms of FADD (FADD-DN) and DAP3 (DAP3-DN), or of NH2-terminal deletion mutant of TRADD (TRADD-ND) achieved by transient transfection experiments, did not impair the kinetics of apoptosis after camptothecin and VP-16 treatment in HL-60 and U937 cells. Taken together, these observations suggested that camptothecin and VP-16 induced rapid aggregation of DR4 and DR3, but paradoxically, the importance of these events in signaling apoptosis is uncertain, because the kinetics of apoptosis were unaffected, even in the presence of a high expression level of SODD, FADD-DN, TRADD-ND, and DAP3-DN. However, camptothecin or VP-16 treatment in combination with tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) substantially accelerated kinetics of apoptosis than treatment with camptothecin, VP-16, or TRAIL alone. In contrast, cotreatment of camptothecin or VP-16 with TWEAK or TL1A did not facilitate apoptosis in HL60 cells. These findings suggest that DR4 aggregation mediated by camptothecin or VP-16 could represent a mean that accelerates TRAIL-induced apoptosis. [Mol Cancer Ther 2004;3(12):1659–69]

Introduction
Chemotherapeutic agents, including DNA topoisomerase I and II inhibitors commonly used in the treatment of hematopoietic tumor cells, could eliminate these cells by rapid activation of apoptosis (1, 2). Apoptosis is triggered by different entry sites, including the best-studied mitochondrial and cell death receptor pathways, the well-described granzyme B pathway in apoptosis by CTL and the less-characterized routes via apical procaspase-2 activation, lysaoptase, and cathepsin activation through lysosomal leakage and calpain activation driven by Ca2+ release from the endoplasmic reticulum (3–7). These pathways lead to sequential activation of a series of cysteine aspartate-specific proteases, called the proteolytic caspase cascade, which results in the orderly death of cells (8).

Several studies have shown that the transcriptional activity of p53 and activation of downstream target genes in response to genotoxic stress, including those provoked by DNA topoisomerase I and II inhibitors, contribute to evoking apoptosis. Among the p53-mediated apoptotic effector genes activated by DNA damage, several pro-apoptotic Bcl-2 family members, including Bax, Noxa, Puma and Bid (9–12), and Peg/Pw1 (13), a group of genes named PIGs (14) and the cell death receptor family members, including FAS, cell death receptor-4 (DR4), and DR5 (15–17), have been recognized as p53-dependent target genes associated with the activation of apoptosis in response to genotoxic stress. Although some of these genes could also be regulated in a p53-independent manner, much less attention has been paid to the p53-independent apoptotic pathways induced by cancer radiotherapy and chemotherapy.

The importance of the cell death receptor family in signaling programmed cell death after genotoxic stress has been the subject of several studies. This family so far includes the membrane death receptors and their respective ligands tumor necrosis factor (TNF)-R1 and TNF-α.
ligand, FAS and FAS ligand, cell death receptor-3 (DR3) and APO-3L/TWEAK and TL1A ligands, DR4 and DR5 that share the TNF-related apoptosis-inducing ligand (TRAIL), and the orphan receptor DR6 (reviewed in ref. 18). Activation of these cell death receptors by their cognate ligands results in receptor trimerization and the formation of high molecular weight death-inducing signaling complexes (DISC). These DISC contain adapter proteins, including FADD, TRADD, DAP3, and a combination of them (e.g., TRADD-FADD for TNF-R1 and DR3, DAP3-FADD for DR4 and DR5, and FADD-only for FAS), which bind to the aggregated receptor through death domain interactions (reviewed in ref. 18). FADD also contains a death effector domain and recruits apical procaspase-8 (or procaspase-10) via death effector domain interactions. Once initiator procaspase-8 zymogen is recruited to the DISC, it begins to cluster and undergoes autoprocessing to generate active caspase-8. In type I cells, procaspase-8 activation at the DISC directly promotes the activation of procaspase-3 and apoptosis in a mitochondria-independent manner (19). However, in most cells, the type II cells, strong activation of procaspase-8 and procaspase-3 requires amplification of the initial signal through the mitochondrial cell death pathway (19). In these type II cells, the BH3-only protein BID is a specific proximal substrate of caspase-8. Cleavage of BID by caspase-8 yields a COOH-terminal BH3-containing fragment that activates downstream mitochondrial apoptotic events (20).

The cell death receptor pathways are also regulated by negative control proteins, including FLICE inhibitory proteins (FLIPs) and silencer of death domains (SODD). FLIPs contains two death effector domains and competes with FADD for binding to procaspase-8 (or procaspase-10), preventing procaspase-8 (or procaspase-10) recruitment and activation at the DISC (21). SODD specifically binds to the death domains of TNF-R1 and DR3, preventing the recruitment of their respective adapter proteins (22). In nonstimulated cells, SODD is believed to be associated with these receptors, inhibiting DISC formation. Upon stimulation by their respective ligands, the dissociation of SODD from the receptors leads to the formation of active DISC (22).

Participation of the cell death receptor family in signaling programmed cell death after anticancer drug treatment had been suggested first by observations in patients with acute myeloid leukemia, where increased FAS expression in blast cells was associated with a better initial response to chemotherapy (23). In some cells, cross-resistance between anticancer drugs and FAS-mediated apoptosis was also noted (24). Together, these findings indicated that drug-induced apoptosis could be mediated by the death receptor FAS signaling pathway, or reflected alterations in common downstream pathways of apoptosis. Several studies have reported that FAS, with or without the activation of its specific ligand, participates in anticancer radiotherapy- and chemotherapy-induced apoptosis in a variety of tumor cell models. Conversely, others proposed that drug-induced apoptosis was independent of the FAS signaling pathway (reviewed in ref. 18). Interestingly, Fulda et al. (25) found that in type I cells, both the FAS signaling system and the mitochondrial pathway participated in triggering apoptosis after drug treatment, whereas in type II cells, apoptosis was predominantly controlled by mitochondria-driven events.

More recently, DR4 and DR5 expression levels and their shared ligand TRAIL have been shown to increase after a variety of DNA-damaging agents, including DNA topoisomerase I and II inhibitors, in a p53-dependent and p53-independent manner. In some of these studies, evidence has been provided for the participation of both cell death receptor– and mitochondria-mediated signaling events triggering apoptosis after drug treatment. In addition, several studies have revealed that combined treatment with a cell death ligand, including FAS ligand and TRAIL, and DNA-damaging agents, including DNA topoisomerase I and II inhibitors, significantly resulted in enhanced synergistic cell death (reviewed in ref. 18). Altogether, these observations supported the idea that such treatment combinations may be useful for clinical cancer therapy. Despite these numerous studies, to the best of our knowledge, the participation of DR3 in drug-induced apoptosis is still unrevealed.

The present experiments were designed to examine the participation of DR3 and DR4, compared with FAS, in p53-deficient leukemia cell lines well described to rapidly undergo apoptosis after DNA topoisomerase I and II inhibitor treatment. We observed that camptothecin and etoposide (VP-16) did not induce an increase in DR3, DR4, and FAS expression but provoked the down-regulation of SODD and FLIPs. Camptothecin promoted strong DR3 aggregation at the cell surface and, to a lesser extent, DR4, whereas VP-16 significantly stimulated DR3 and DR4 aggregation. However, the high expression level of SODD or of dominant negative forms of FADD (FADD-DN) and DAP3 (DAP3-DN), or of NH2-terminal deletion mutant of TRADD (TRADD-DN) achieved in transient transfection experiments, did not slow the kinetics of apoptosis in camptothecin- or VP-16-treated HL60 and U937 cells. Camptothecin or VP-16 treatment in combination with TRAIL substantially accelerated the kinetics of apoptosis than treatment with camptothecin, VP-16 or TRAIL alone. In contrast, cotreatment of camptothecin or VP-16 with TWEAK or TL1A did not accelerate apoptosis in HL60 cells. Our results suggest that the importance of DR3 and DR4 in signaling apoptosis in HL60 and U937 cells after camptothecin or VP-16 treatment is uncertain, but camptothecin or VP-16 could accelerate TRAIL-, but not TWEAK- or TL1A-induced apoptosis in HL60 cells.

Materials and Methods

Reagents

The radioactive precursors [2,14C]-thymidine (59 mCi/ mmol) was obtained from ICN BioMedicals (Costa Mesa, CA). Camptothecin and VP-16 were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant hTRAIL was

Chemical Co. (St. Louis, MO). Recombinant hTRAIL was
from Calbiochem-Novabiochem Co. (San Diego, CA), and recombinant hTWEAK and hTL1A were obtained from R&D Systems (Minneapolis, MN). The fluorogenic peptide substrates acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (Ac-DEVD-AMC), benzoyloxycarbonyl-Ile-Asp-Thr-Asp-7-amino-4-trifluoro methylcoumarin (z-IETD-AFC), and acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl-coumarin (Ac-LEHD-AMC) were provided from Bachem Bioscience, Inc. (King of Prussia, PA) or Calbiochem-Novabiochem. Rabbit polyclonal anti-human caspase-8 (559932) and DR4 (66891N), and mouse monoclonal anti-human TRADD (B36-2) and FADD (A66-2) antibodies were purchased from Pharmingen, Inc. (San Diego, CA). Rabbit polyclonal anti-human FAS (sc-715) and SODD (sc-8980) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-human DR3 (06-839) and mouse monoclonal anti-FLAG (M2) were from Upstate Biotechnology (Lake Placid, NY), rabbit polyclonal anti-human FLIP/γ/δ (343006) from Calbiochem-Novabiochem and mouse monoclonal anti-HA (clone 12CA5) from Roche Diagnostics (Laval, Que). All other chemicals and reagents were acquired from Sigma Chemical, ICN, or local sources.

**Cell Culture, cDNA Cloning, Transfection, DNA Labeling, and Drug Treatments**

The human HL60 and U937 cell lines, from the American Type Culture Collection (Manassas, VA), were grown in suspension culture at 37°C under 5% CO2 in a humidified atmosphere in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin. The expression vectors pRK-FLAG-SODD and pcDNA3-GFP-FADD (80–208) were kind gifts from Drs. W. Liu (Tularik, Inc., San Francisco, CA; ref. 22) and H. Wajant (Institut fuer Zelbiologie und Immunologie, Stuttgart, Germany; ref. 26), respectively. From the expression vectors pRK-myc-TRADD obtained from Dr. Goeddel (Tularik; ref. 27), we generated a NH2-terminal deletion mutant of TRADD (TRADD-ND; ref. 28) lacking the NH2-terminal 1 to 108 amino acids, by PCR using specific adapter primers containing BamHI sequences. The DAP3-DN cDNA that lacks the COOH-terminal 231 to 398 amino acids (29), was generated by reverse transcription-PCR from polyA(+)-RNA extracted from HL60 cells, using specific adapter primers containing NotI sequences. The amplified fragment was first cloned in pCR2.1 TOPO vector (TA cloning system; Invitrogen, San Diego, CA) and then subcloned at the BamHI or NotI restriction sites in the eukaryotic expression vector pCEP4 (Invitrogen, Carlsbad, CA) that has been modified to include hemagglutinin epitope Tag sequences (HA-tag) and Kozak consensus sequences (30). All transfections were done by electroporation at 0.27 kV (Gene Pulser, Bio-Rad, Hercules, CA) with 10 µg of each plasmid and 10 × 10⁶ cells. For DNA-labeling, cells were grown with [3H]-thymidine (0.02 Ci/mL) for 24 hours and chased overnight in isotope-free medium prior to electroporation or drug treatment. Exponentially growing cells (5 × 10⁵ cells/mL) were used for camptothecin (1.0 mol/L) or VP-16 (50 mol/L) treatment. After 30 minutes incubation with the drugs, the cells were centrifuged and resuspended in drug-free medium. TRAIL (250 ng/mL), TWEAK (500 ng/mL), and TL1A (500 ng/mL) treatments were done during and after camptothecin or VP-16 exposure.

**Caspase Activity Determinations**

Control and drug-treated cells were homogenized at 4°C for 30 minutes in lysis buffer containing 100 mmol/L HEPES (pH 7.5), 5 mmol/L EDTA, 5 mmol/L DTT, 20% glycerol, and 0.1% NP40. The samples were centrifuged (10,000 × g for 10 minutes at 4°C), and supernatants collected as cytosolic extracts. Caspase activity was measured by monitoring fluorescence continuously in a dual luminescence LS 50B fluorometer (Perkin-Elmer, Buckinghamshire, UK) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm for the substrate Ac-DEVD-AMC and at an excitation wavelength of 400 nm and an emission wavelength of 505 nm for the substrates z-IETD-AFC and Ac-LEHD-AMC. Reactions were conducted in cuvettes, and temperature was maintained at 37°C with a water-jacketed sample compartment. DEVDase and IETDase activities were studied in an optimal reaction buffer (31) containing 100 mmol/L HEPES (pH 7.5), 100 mmol/L NaCl, 10% glycerol, 0.1% CHAPS, 10 mmol/L DTT, 1 mmol/L EDTA, and 200 µmol/L fluorogenic peptide substrates. LEHDase activity was measured in a reaction buffer containing 100 mmol/L MES (pH 6.5), 10% FEG8000, 0.1% CHAPS, 10 mmol/L DTT, 1 mmol/L EDTA, and 500 µmol/L Ac-LEHD-AMC (31). Enzyme activities were determined as initial velocities (relative intensity minute⁻¹ mg⁻¹) and the results are expressed as fold-increase relative to control, untreated cells.

**DNA Fragmentation Assays**

To visualize oligonucleosome-sized DNA fragmentation, cellular DNA was extracted and DNA electrophoresis was done on 1.6% agarose gel in Tris-acetate buffer (pH 8.0) prior to ethidium bromide staining (2). A DNA filter elution assay was used to quantitate DNA fragmentation, as described previously (2). The results were expressed as the percentage of DNA fragmented in treated cells compared with DNA fragmented in untreated cells (background), using the formula (F – F₀ / F₀ × 100), where F and F₀ represent DNA fragmentation in treated and control cells, respectively.

**Indirect Immunofluorescence and Immunoperoxidase Staining**

Approximately 1.0 × 10⁵ control and drug-treated cells were spread by cytocentrifugation on glass slides and fixed in ice-cold ethanol for 10 minutes and air-dried for 10 minutes. For immunofluorescence microscopy, nonspecific binding sites were blocked with 5% bovine serum albumin in the presence of irrelevant antibodies (antimouse immunoglobulin G, 15 µg/mL; Vector Laboratories, Birmingham; CA); then the slides were washed in PBS and incubated with the primary antibody (10 µg/mL) for 1 hour at room temperature. After several washes in PBS, the slides were probed with anti-rabbit FITC-conjugated secondary antibodies (10 µg/mL; Vector Laboratories) for 1 hour at
room temperature. For immunoperoxidase assay, endogenous peroxidase activities were blocked with 3% H2O2 solution for 15 minutes. The slides were then probed and stained with Vectastain ABC Elite Kit reagents, according to the manufacturer’s protocols (Vector Laboratories). Images were generated with a Nikon Optiphot-2 microscope equipped with a thermoelectrically cooled CCD camera (Model DC330E, DageMTI, Inc., Michigan City, IN) hooked up to a PC computer. All images were analyzed with Cлемекс Vision software (version 3.0.036, Cлемекс, Longueuil, Quebec, Canada).

Protein Expression by Western Blot Analysis

For Western blot analysis, cells were collected and homogenized in lysis buffer containing 5 mmol/L HEPES (pH 7.4), 160 mmol/L KCl, 40 mmol/L NaCl, 10 mmol/L MgCl2, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, 0.5% NP40, and a cocktail of protease inhibitors (Complete, Roche Molecular Biochemicals, Montreal, Quebec, Canada) at 4°C for 30 minutes. After centrifugation (10,000 × g, 10 minutes), the supernatants were collected as protein extracts. Proteins were loaded for SDS-PAGE and electroblotted on nitrocellulose membranes. Immunoblots were revealed by enhanced chemiluminescence reagent (Amersham Life Science, Piscataway, NJ). Relative densitometry analysis of film exposure was based on integrated density values using an Alpha Imager 2000 digital imaging system (Alpha Innotech Co., San Jose, CA).

Gel Filtration Column Chromatography

Gel filtration chromatography was done on a Superose 6HR 16/50 column connected to a fast protein liquid chromatography system (Amersham Life Science). The equilibration, lysis and elution buffer contained PBS, 1% NP40, and a cocktail of protease inhibitors (Complete, Roche Molecular Biochemicals). Cellular extracts were prepared from 5 × 106 cells/mL volume. After centrifugation, soluble extracts (500 μL) were injected onto a column for elution at a flow rate of 500 μL/min, and fractions were collected at 5-minute intervals. The corresponding molecular weights and Stokes’ radius for each standard (Amersham Life Science) were RNase A (13.7 kDa, 16 Å), chymotrypsinogen A (25 kDa, 20.9 Å), ovalbumin (43 kDa, 30.5 Å), albumin (67 kDa, 35.5 Å), aldolase (158 kDa, 48.1 Å), catalase (232 kDa, 52.2 Å), apoferitin (440 kDa, 61.0 Å), and thyroglobulin (669 kDa, 85 Å). Void volume corresponded to the elution of Blue Dextran 2000. After column chromatography, 80-μL aliquots from each fraction were analyzed by SDS-PAGE and Western blotting.

Results

Aggregation at the Cell Surface of DR3 and DR4 after Camptothecin and VP-16 Treatment in HL60 Cells

The kinetics of camptothecin- and VP-16-induced apoptosis in HL60 cells were reported previously (1, 2). Short drug treatments (30 minutes) with camptothecin or VP-16 rapidly induced caspase activation, including DEVDase (caspase-3 like), IETDase (caspase-8 like), and LEHDase (caspase-9 like) activities (Fig. 1A), associated by DNA fragmentation. This DNA fragmentation was quantitated by DNA filter elution assay and visualized as oligonucleosome-sized DNA ladders on ethidium bromide-stained agarose gels (Fig. 1B). Rapid activation of IETDase (caspase-8 like) and LEHDase (caspase-9 like) activities could suggest the participation of both the cell death receptor and mitochondrial pathways, to signal apoptosis after camptothecin and VP-16 treatment in HL60 cells. However, several studies have revealed the existence of cell death receptor-independent mechanisms for procaspase-8 activation (32, 33).

To more directly evaluate the participation of DR3, DR4 and FAS in signaling caspase activation after camptothecin or VP-16 treatment, we first looked for death receptor aggregation at the cell surface by indirect immunofluorescence and immunoperoxidase staining (Fig. 2). Found primarily as monomeric proteins in resting cells, these receptors, upon activation, aggregate into high molecular weight complexes (34) that can be visualized as dense, patchy clusters, and aggregates by indirect immunofluorescence staining (35). Exposure of HL60 cells to camptothecin or VP-16 failed to induce large and dense staining aggregates of FAS after indirect immunofluorescence (Fig. 2, top) or immunoperoxidase staining (Fig. 2, bottom). In contrast, DR3 immunoreactivity was detected as dense and brightly stained clusters after camptothecin or VP-16 treatment (Fig. 2). Similarly, DR4 was visualized as dense patchy staining after VP-16 treatment, and slightly weaker after camptothecin treatment (Fig. 2).

To evaluate if DR3 and DR4 aggregation resulted from an increase in their expression after camptothecin or VP-16 treatment, the kinetics of their expression were analyzed by Western blotting and immuncyttofluorometry. As shown in Fig. 3, the expression level of DR3, DR4, and FAS remained mostly unchanged for at least 8 hours after camptothecin or VP-16 treatment in HL60 cells. The variations observed in some experiments were not significantly different (Student’s t test: P > 0.05). In contrast, the FLIPS expression level decreased after camptothecin or VP-16 treatment, with the most rapid and pronounced effect occurring after VP-16 (Student’s t test: P < 0.008 for camptothecin at 8 hours and P < 0.001 for VP-16 at 4, 6, and 8 hours). Similarly, the SODD expression level also declined significantly after camptothecin or VP-16 treatment (Student’s t test: P < 0.001 for camptothecin at 4, 6, and 8 hours; P < 0.008 for VP-16 at 4 hours; and P < 0.001 at 6 and 8 hours; Fig. 3). To confirm some of these observations, the total protein amount of DR3, DR4, and FAS was also measured by indirect immunocytofluorometry in permeable cells. The fluorescence intensity distribution detected for DR3, DR4, and FAS remained mostly unchanged in the absence of and after camptothecin or VP-16 treatment (data not shown). Altogether, these observations indicated that DR3 and DR4 aggregation on the cell surface did not result from an increased expression.
of these receptors, because a comparable level of proteins was detected in untreated and treated HL60 cells. In addition, the decreased expression of two inhibitors of the death receptor signaling pathways after camptothecin or VP-16 treatment suggests the participation of these cell death receptor signaling pathways in apoptosis activation and/or amplification in the cells.

**Formation of High Molecular Weight Complexes Involving DR3 and DR4 after Camptothecin and VP-16 Treatment**

Initial attempts failed to immunoprecipitate the DR3 and DR4 death-inducing signaling complexes after camptothecin or VP-16 treatment in HL60 cells, in the absence of added ligands. To explore the formation of these complexes, a gel filtration column chromatography approach, followed by Western blot analysis of the multiple collected fractions, was chosen. This approach allowed us to separate and monitor proteins of interest within a large range of molecular masses, revealing multigolomer and complex formation.

In untreated control HL60 cells, the elution profiles of DR3 and DR4 revealed a large distribution of these proteins from low to very high molecular weight masses, whereas the expression profile of FAS showed a narrower distribution (Fig. 4A). In camptothecin- or VP-16-treated cells, at 2 and 3.5 hours after treatment, the elution profiles of DR3 shifted and concentrated toward fractions 26 to 28 corresponding to molecular weight masses ranging approximately from 500 to 300 kDa. The elution profiles of DR4, at 2 and 3.5 hours after VP-16 treatment, exhibited similar behavior, shifting and concentrating in a few fractions (24–26) corresponding to high molecular weight masses ranging from ~700 to 500 kDa. In contrast, the elution profiles of DR4 after camptothecin treatment did not show striking differences compared with control cells. Similarly, the elution profiles of FAS after camptothecin or VP-16 treatment remained mostly unchanged compared with control cells (Fig. 4A).

We next monitored the elution profiles of two adapter proteins, TRADD and FADD, associated with DR3 or DR4 death-inducing signaling complexes. Neither TRADD nor FADD coeluted in fractions with DR3 (fraction 26–28) or
DR4 (fraction 24–26) after camptothecin or VP-16 treatment (Fig. 4B). Similarly, we monitored the elution profiles of SODD, a specific inhibitory protein of DR3, and FLIPS, an inhibitor of both DR3 and DR4 death-inducing signaling complexes. Whereas FLIPS did not coelute in fractions with DR3 or DR4, SODD showed strong coelution profiles with DR3 at 2 and 3.5 hours after camptothecin treatment, and in some fractions 3.5 hours after VP-16 treatment (Fig. 4C). Finally, the elution profiles of procaspase-8 were distinct than those of DR3 and DR4 after camptothecin treatment, but a few amount of procaspase-8 coeluted with DR3 and/or DR4 at 3.5 hours after VP-16 treatment. The cleaved fragments of active caspase-8 were also detected after camptothecin and VP-16 treatment (Fig. 4D).

Altogether, these observations were consistent with the immunofluorescence and immunoperoxidase staining experiments and indicated that DR3, and DR3 and DR4 formed oligomers and aggregates of high molecular weight after camptothecin and VP-16 treatment, respectively. The effect of camptothecin on DR4 aggregation remains enigmatic. Whereas some patchy staining after camptothecin treatment was observed by indirect immunofluorescence and immunoperoxidase imaging, no shift and cluster formation of high molecular weight involving DR4 were detected by gel filtration column chromatography after camptothecin. Under the conditions of these experiments, we were also unable to observe coelution of the adapter proteins TRADD and FADD within the DR3 and DR4 complexes, suggesting that the aggregates were not formed by typical active DISC components.

Figure 2. Effect of camptothecin and VP-16 treatment on FAS, DR3, and DR4 aggregation at the cell surface of HL60 cells. Cells were untreated (CONTROL, left) or treated with camptothecin (1.0 μmol/L, middle) or VP-16 (50 μmol/L, right) for 30 minutes followed by incubation in drug-free medium. At 4 hours after treatment, FAS, DR3, and DR4 aggregation was visualized by indirect immunofluorescence (top) and immunoperoxidase (bottom) staining. Images were generated with a Nikon Optiphot-2 microscope equipped with a thermoelectrically cooled CCD camera (model DC330E).
Conversely, some procaspase-8 coeluted with DR3 and/or DR4 after VP-16 treatment, suggesting the formation of weak DR3-procaspase-8- and/or DR4/procaspase-8-containing complexes. Interestingly, we found a strong coelution association between SODD and DR3, suggesting the presence of this death inhibitory protein within the DR3 complexes predominantly after camptothecin treatment.

Figure 3. Kinetics of FAS, DR3, DR4, FLIPs, and SODD expression in camptothecin- and VP-16-treated HL60 cells. Cells were treated with camptothecin (1.0 μmol/L) or VP-16 (50 μmol/L) for 30 minutes followed by incubation in drug-free medium. At the indicated times after camptothecin (left) or VP-16 (right) treatment, Western blot analysis was done. Typical film exposures and relative densitometry analysis done in triplicate of n experiments. ●, data distribution; open column, means; bars, ±SD; filled columns, means; bars, ±SE; P values, Student’s t test. Differences with P < 0.05 were considered significant. CRK-L expression as loading control.

Figure 4. High molecular weight complex formation in camptothecin- and VP-16-treated HL60 cells. Cells were treated with camptothecin (1.0 μmol/L) or VP-16 (50 μmol/L) for 30 minutes followed by incubation in drug-free medium. Cell extracts prepared from control and drug-treated cells at 2 and 3.5 hours after treatment were subjected to gel filtration chromatography on a Superose 6HR 16/50 column (flow rate, 500 μL/min). Fractions of 2.5 mL were collected (number above), and 80-μL aliquots of each fraction were analyzed by SDS-PAGE and Western blotting. Arrows, elution profiles of the molecular weight standard proteins. A, elution profiles of DR3, DR4, and FAS. B, elution profiles of TRADD and FADD. C, elution profiles of SODD and FLIPs. D, elution profile of procaspase-8 and caspase-8 fragments.
SODD, FADD-DN, TRADD-ND, and DAP3-DN Overexpression Did Not Impair Camptothecin and VP-16-Induced Apoptosis in HL60 and U937 Cells

From the above experiments, it was difficult to associate or not a functional role for the DR3 and DR4 high molecular weight aggregates in signaling caspase activation and apoptosis after camptothecin or VP-16 treatment. Whereas all or part of the DR3 complexes could be inhibited by the presence of SODD, no adapter or inhibitory proteins were found to be associated with the DR3 or DR4 clusters, but a low amount of procaspase-8 was detected in high molecular weight fractions with DR3 and/or DR4 after VP-16 treatment. To further elucidate these questions, we examined the consequences of overexpressing SODD, FADD-DN, TRADD-ND, and DAP3-DN on camptothecin- and VP-16-induced apoptosis. Figure 5A shows representative expression level of these proteins achieved in transient transfection experiments on HL60 cells. The high expression level of SODD, FADD-DN, TRADD-ND, and DAP3-DN did not impair the kinetics of DNA fragmentation (Fig. 5B) and caspase activation, including IETDase (caspase-8 like), LEHDase (caspase-9 like), and DEVDase (caspase-3 like) activities (data not shown), after camptothecin or VP-16 treatment. Similar results were obtained in U937 cells (Fig. 5C and D). These results revealed that the importance of DR3 and DR4 aggregation to signal apoptosis in these cells after camptothecin or VP-16 treatment is uncertain, or at least not linked with the death-inducing signaling pathways inhibited by a high expression level of SODD, FADD-DN, TRADD-ND, and DAP3-DN.

Combination Treatment of Camptothecin or VP-16 with TRAIL, TWEAK, or TL1A

Several reports have shown that chemotherapeutic agents increase FAS- or TRAIL-induced apoptosis, associated or not with up-regulation of FAS, DR4 or DR5 (reviewed in ref. 18). Thus, to investigate a functional significance for camptothecin- and VP-16-mediated DR3 and DR4 aggregation, we examined the kinetics of apoptosis in HL60 cells treated with TRAIL, the DR4 ligand, and TWEAK or TL1A, described as putative DR3 ligands, alone or in combination with camptothecin or VP-16. Figure 6 shows that camptothecin (1.0 μmol/L) or VP-16 (50 μmol/L) treatment in combination with TRAIL (250 ng/mL) substantially accelerated kinetics of apoptosis than treatment with camptothecin, VP-16, or TRAIL alone. In contrast, cotreatment of camptothecin (1.0 μmol/L) or VP-16 (50 μmol/L) with TWEAK (500 ng/mL), or TL1A

---

**Figure 5.** Effect of FADD-DN, TRADD-ND, DAP3-DN, and SODD overexpression on camptothecin- and VP-16-induced apoptosis in HL60 and U937 cells. HL-60 (A and B) and U937 (C and D) cells were transfected by electroporation with control vector, GFP-FADD-DN, HA-TRADD-ND, DAP3-DN, or FLAG-SODD. Transfection efficiency was determined by measuring GFP by flow cytometry (≥70%). After 24 hours, cells were treated with camptothecin (1.0 μmol/L) or VP-16 (50 μmol/L) for 30 minutes followed by incubation in drug-free medium. A and C, representative expression level of GFP-FADD-DN, HA-TRADD-ND, DAP3-DN, or FLAG-SODD in HL60 (A) and U937 (C) cells 24 hours after transfection. B and D, at the indicated times (x axis, h) after camptothecin or VP-16 treatment, kinetics of DNA fragmentation were determined by DNA filter elution assays. B, points, mean ± range of two independent experiments. D, points, mean ± range of two independent experiments.
Figure 6. Cotreatment of camptothecin or VP-16 with TRAIL, TWEAK, or TL1A in HL60 cells. [2-14C]-Thymidine-labeled cells were treated with (A) 250 ng/mL TRAIL, (B) 500 ng/mL TWEAK, and (C) 500 ng/mL TL1A alone or in combination with 1.0 μmol/L camptothecin or 50 μmol/L VP-16. Kinetics of DNA fragmentation, at various time points (x axis, h), was determined by DNA filter elution assay. Points, mean % DNA fragmentation (n = 4 in A, n = 3 in B and C); bars, ±SE. ■, camptothecin or VP-16 alone; ▲, TRAIL, TWEAK, or TL1A alone; ◆, camptothecin or VP-16 in combination with TRAIL, TWEAK, or TL1A.

Discussion
In the present study, we examined the participation of DR3 and DR4 in signaling apoptosis after DNA topoisomerase I and II inhibitor treatment in HL60 and U937 cells. Several studies have suggested the participation of cell death receptor family members, including FAS, DR4, or DR5, with or without the activation of their specific ligands, in apoptosis induced by radiotherapy and chemotherapy in a variety of cancer cells. In contrast, others have reported that genotoxic stress-induced apoptosis could be independent of the cell death receptor signaling pathways (reviewed in ref. 18). From these studies, it seemed that in some cells, both a cell death signaling system involving FAS, DR4, or DR5 and the mitochondrial pathway participated in triggering apoptosis after drug treatment, whereas in other cells, apoptosis was predominantly controlled by mitochondria-driven events.
In this study, we observed, by indirect immunofluorescence and immunoperoxidase staining, the rapid aggregation and oligomerization of DR3 and DR4, but not FAS, in HL60 cells treated with camptothecin or VP-16. Using gel filtration column chromatography, we confirmed the aggregation of DR3 after camptothecin and VP-16, and of DR4 only after VP-16 treatment. The molecular mechanisms underlying DR3 and DR4 aggregation after camptothecin or VP-16 treatment are still unknown. It is generally believed that the death domains of these receptors can self-associate upon stimulation by their respective ligands or independently of ligands when highly expressed. No increase in DR3 or DR4 and FAS expression was observed after camptothecin or VP-16 treatment in HL60 cells. Although activation and the expression level of their respective ligands were not measured in this study, earlier reports have revealed that neither FAS nor its ligand was up-regulated in doxorubicin-treated HL60 cells (36), and that DR4 and TRAIL levels did not increase in VP-16- or doxorubicin-treated HL60 cells (37).

SODD, the silencer of death domain-containing receptors, including TNF-R1 and DR3 (22), showed strong coelution profiles in fractions with DR3, predominantly after camptothecin, and slightly after VP-16 treatment, by gel filtration column chromatography. These elution profiles suggested that the association between DR3 and SODD could prevent the recruitment and binding of the adapter proteins TRADD and FADD, blocking the formation of active death-inducing signaling complexes. Moreover, the expression level of SODD decreased significantly at 4 hours after camptothecin and VP-16 treatment in HL60 cells, raising the possibility that DR3 could be released from the blocking effect of SODD. Such release could eventually initiate the formation of active death-inducing signaling complexes that would participate in signaling apoptosis. To further elucidate if decreased SODD expression could have an effect on the kinetics of caspase activation and apoptosis induced by camptothecin and VP-16, a high expression level of SODD was achieved in transient transfection experiments. In these experiments, a high expression level of SODD in HL60 and U937 cells did not impair or slow the kinetics of apoptosis after camptothecin and VP-16 treatment. These results indicated that the aggregation of DR3 and down-regulation of SODD were neither absolutely required nor strongly linked with the kinetics of apoptosis in these cells after camptothecin or VP-16 treatment.

Similarly, the FLIPS expression level decreased significantly after VP-16 treatment in HL60 cells. Although FLIPS did not coelute by gel filtration column chromatography in fractions with DR4 or DR3, it was found in similar fractions with the adapter protein FADD and TRADD and with procaspase-8. FLIPS contains two death effector domains and competes between adapter proteins and procaspase-8, preventing the formation of active DISC (21). To further elucidate if decreased FLIPS expression could have an effect on camptothecin- or VP-16-induced caspase activation and apoptosis, we used FADD-DN that has a similar but more dominant effect compared with FLIPS in preventing the formation of active DISC. Also, similar experiments were done with other adaptor proteins, including DAP3-DN and TRADD-DN. High expression levels of FADD-DN, DAP3-DN, and TRADD-DN achieved in transient transfection experiments did not inhibit the kinetics of DNA fragmentation after camptothecin and VP-16 treatment in HL60 and U937 cells. Altogether, these results are in agreement with previous observations indicating that the presence of a neutralizing anti-FAS immunoglobulin G antibody failed to inhibit both doxorubicin- and VP-16-induced apoptosis in HL60 cells (36), and that HL60 cells may be like type II cells with respect to the importance of cell death receptor-initiated apoptotic signaling after chemotherapy (25, 37).

Although, a recruitment and activation of cell death receptor/ligand systems are not absolutely required for radiotherapy- and chemotherapy-induced apoptosis in a variety of tumor cells, including the HL60 cell line (this study and refs. 36, 37), many reports have revealed that combined cell death ligand and drug treatment produces a synergistic cytotoxic effect, which may prove useful in the treatment of various human tumors, including leukemia and lymphoma (reviewed in ref. 18). These effects were also observed in CEM and HL60 cells, where low-dose agonistic anti-FAS IgM antibodies or TRAIL ligand in combination with DNA topoisomerase I or II inhibitors, elicited a synergistic cytotoxic effects, although neither FAS and its ligand nor DR4 and TRAIL were found to be up-regulated in these cells (36, 37). Similar observations were made in our study, where camptothecin or VP-16 treatment in combination with TRAIL, substantially accelerated kinetics of apoptosis than treatment with camptothecin, VP-16 or TRAIL alone. Taken together, our results and those of others (36, 37) suggest that, even in the absence of increased expression levels, some of these cell death receptors rapidly aggregate at the cell surface after camptothecin or VP-16 treatment. In turn, such aggregation may set or sensitize these cells to low-dose cell death ligand treatment.

In this study, we also observed that the less-characterized DR3 rapidly aggregated at the cell surface and that the SODD expression level decreased after drug treatment. Two ligands have been proposed to bind DR3, including APO-3L/TWEAK and TL1A, although some controversial data have emerged in the past concerning their specificity (38–43). In our study, treatment with TWEAK or TL1A alone did not induce apoptosis in HL60 cells, and cotreatment with camptothecin or VP-16 did not accelerate kinetics of apoptosis than treatment with camptothecin or VP-16 alone. Thus, our data support those previous observations suggesting that TWEAK or TL1A are poor death ligand of DR3.

In conclusion, camptothecin- and VP-16-induced apoptosis in human leukemia HL60 and U937 cells is independent of DR3 and DR4 signaling pathways. However, camptothecin or VP-16 substantially accelerate TRAIL-α, but not TWEAK- or TL1A-induced apoptosis in HL60 cells.
Acknowledgments

We thank Drs. W. Liu and D.V. Goeddel (Tularik) for the pRF-FLAG-SODD and pRF-Myc-TRADD vectors, H. Wajant (Institut fuer Zelbiologie und Immunologie) for pcDNA3-GFP-FADD (80–208) vector, and the editorial work of Ovid Da Silva (Research Support Office, Research Centre of the Centre hospitalier de l’Universite de Montreal Hospital).

References

21. Tschopp J, Irmler M, Thome M. Inhibition of Fas death signals by FLIPs. Curr Opin Immunol 1998;10:552–8.
Molecular Cancer Therapeutics

Camptothecin- and etoposide-induced apoptosis in human leukemia cells is independent of cell death receptor-3 and -4 aggregation but accelerates tumor necrosis factor–related apoptosis-inducing ligand–mediated cell death

Stephane Bergeron, Myriam Beauchemin and Richard Bertrand


Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/3/12/1659

Cited articles
This article cites 41 articles, 17 of which you can access for free at:
http://mct.aacrjournals.org/content/3/12/1659.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.