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Tepper, A.D.

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Jeanine G.R. Boesen-de Cock
Annemiek D. Tepper
Evert de Vries
Wim J. van Blitterswijk
Jannie Borst

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Common Regulation of Apoptosis Signaling Induced by CD95 and the DNA-damaging Stimuli Etoposide and γ-Radiation Downstream from Caspase-8 Activation*

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Jeanine G. R. Boesen-de Cock, Annemiek D. Tepper, Evert de Vries, Wim J. van Blitterswijk, and Jannie Borst†

From the Division of Cellular Biochemistry, Netherlands Cancer Institute, Plesmanalaan 121, 1066 CX Amsterdam, The Netherlands

The death receptor CD95 (APO-1/Fas), the anticancer drug etoposide, and γ-radiation induce apoptosis in the human T cell line Jurkat. Variant clones selected for resistance to CD95-induced apoptosis proved cross-resistant to etoposide- and radiation-induced apoptosis, suggesting that the apoptosis pathways induced by these distinct stimuli have critical component(s) in common. The pathways do not converge at the level of CD95 ligation or caspase-8 signaling. Whereas caspase-8 function was required for CD95-mediated cytochrome c release, effector caspase activation, and apoptosis, these responses were unaffected in etoposide-treated and irradiated cells when caspase-8 was inhibited by FLIP(L). Both effector caspase processing and cytochrome c release were inhibited in the resistant variant cells as well as in their transfectants, suggesting that, in Jurkat cells, the apoptosis signaling pathways activated by CD95, etoposide, and γ-radiation are under common mitochondrial control. All three stimuli induced ceramide production in wild-type cells, but not in resistant variant cells. Exogenous ceramide bypassed apoptosis resistance in the variant cells, but not in Bcl-2-transfected cells, suggesting that apoptosis signaling induced by CD95, etoposide, and γ-radiation is subject to common regulation at a level different from that targeted by Bcl-2.

Cells can undergo apoptosis in response to a broad spectrum of stimuli, including receptor stimulation, treatment with cytotoxic drugs, and γ-radiation. Although a given stimulus may activate unique signaling molecules, the current model states that the molecular events in the execution phase of apoptotic cell death are shared. Members of the caspase family, which are aspartate-specific cysteine proteases, are key initiators of this execution phase (1, 2). Their proteolytic action on specific cellular components, including other caspases, structural proteins, and enzymes, leads to the ordered degradation of the cell into apoptotic bodies.

Certain members of the tumor necrosis factor receptor family, including CD95, directly couple to the caspase cascade via their cytoplasmic death domain. Upon multimerization by its trimeric ligand, CD95 recruits the FADD adaptor via its death domain (3). Via its death effector domain (DED), FADD, in turn, recruits caspase-8 (FLICE), which contains two related DEDs in its amino terminus (4, 5). Recently, several viral proteins, termed v-FLIP (FLICE inhibitory protein), were identified that contain two regions of homology to the DEDs of FADD and caspase-8 (6–8). Mammalian cells express FLIP homologues: FLIP<sub>B</sub>, composed of two DEDs, and FLIP<sub>L</sub>, which contains, in addition, a nonfunctional caspase domain (9–11). Both viral and cellular FLIP proteins bind to FADD and/or caspase-8 and inhibit death receptor-induced apoptosis, most likely by displacing DED-containing caspases (caspase-8 and/or -10) from the activated death receptor complex (8–10).

Whereas it is to some extent understood how death receptors link to the caspase family, this is less clear for other apoptotic stimuli. In certain cases, the stimulus indirectly activates death receptors. An example is the T cell antigen receptor, which induces synthesis of CD95 ligand and therewith activates CD95 in, for example, Jurkat T cells (12). A similar mechanism has been proposed for apoptosis induction by anticancer drugs (13, 14). Apart from the death-inducing signaling complex (DISC) at the death receptor cytoplasmic tail (4, 15, 16), the mitochondrial membrane is now thought to be a site for initial caspase activation. In response to various apoptotic stimuli, mitochondria release cytochrome c (17–19), which, together with Apaf-1 (20) and caspase-9 (21), can activate caspase-3 in vitro. The Apaf-1-caspase complex is thought to be located at the mitochondrial membrane since the homologous Caenorhabditis elegans proteins CED-4 and CED-3 form a complex together with CED-9, a homologue of mammalian Bcl-2 (22, 23) that resides at this site (24). Mitochondrial cytochrome c release can be blocked by the apoptosis inhibitory proteins Bcl-2 and Bcl-xL by an unknown mechanism (17, 18).

Many apoptosis pathways, including those induced by etoposide and γ-radiation, are controlled by the Bcl-2 family, suggesting that, in these cases, mitochondrial participation is essential for the cell death response (25, 26). Etoposide inhibits topoisomerase II and therewith induces double strand DNA breaks (27), whereas γ-radiation also induces DNA damage. Both stimuli activate caspases (28–30), but it is unknown how the signal is transmitted to these enzymes. In certain cell types, DNA damage induces p53-directed de novo synthesis of the Bcl-2 antagonist Bax (31), indicating a mechanism for regulation of the mitochondrial caspase pathway. It has also been shown that anticancer drugs can induce expression of CD95 and/or its ligand and therewith activate the CD95 pathway (13, 14). However, this is not a general mechanism since etoposide-induced apoptosis is CD95-independent in murine death-inducing signaling complex; mAb, monoclonal antibody; FLIP, FLICE inhibitory protein; PIPES, 1,4-piperazineethanesulfonic acid; IR, γ-radiation; Gy, gray.
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thymocytes and Jurkat cells (32).

Several studies suggest that the lipid ceramide is relevant for apoptosis signaling in response to death receptors, γ-irradiation, and anticancer drugs. Tumor necrosis factor receptor-1 (33); CD95 (34); γ-radiation (35); and daunorubicin, vincristine, and cytosine arabinoside (36-39) can induce accumulation of ceramide. Moreover, failure to generate ceramide has been associated with apoptosis resistance (34, 37, 40, 41). Exogenous short-chain ceramide can induce apoptosis in certain cell types, but it is not clear whether it is equivalent in its action to naturally generated ceramide. The ceramide response induced by CD95 lies upstream from DEVD-inhibitable caspases and naturally generated ceramide. The ceramide response induced by CD95 lies upstream from DEVD-inhibitable caspases and nuclear segmentation (42), allowing for the possibility that ceramide contributes to generation of the apoptotic phenotype.

Jurkat cells selected for resistance to CD95-induced apoptosis were found to be cross-resistant to etoposide and γ-radiation. Therefore, we have examined possible convergence of the apoptosis signaling pathways induced by these three stimuli. We find that the pathways share features downstream from CD95 and the DNA-damaging regimens. Thus we have examined possible convergence of the apoptosis signaling pathways induced by these three stimuli. We find that the pathways share features downstream from CD95 and the DNA-damaging regimens.

EXPERIMENTAL PROCEDURES

Reagents—(1,14C)Serine (54.0 mCi/mmol) and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Pharmacia Biotech. Anti-CD95 mAb was from Immunotech (Marseille, France). Anti-caspase-8 serum was raised in rabbits against a synthetic peptide comprising amino acids 2–20 of human caspase-8. Specificity of the antisera was confirmed as described previously (42, 43). Mouse anti-human caspase-3 mAb was purchased from Transduction Laboratories (Lexington, KY); mouse anti-human caspase-6 (B93-4) and caspase-7 (B94-1) mAbs and anti-cytochrome c mAb THS.2C12 were from Pharmingen; and anti-cytochrome c mAb C4 was from Roche Molecular Biochemicals. Horseradish peroxidase-conjugated rabbit anti-mouse Ig G was from Dako A/S (Glostrup, Denmark).

Cells—The J15 wild-type clone was derived from the human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution and selection (32, 42). CD95-resistant JA variant clones were derived by limiting dilution from the Jurkat line, cultured for 5 weeks in the presence of 1 μg/ml anti-CD95 mAb (44). Clonogenic assays indicated that ~2 in 1000 cells of the parental line survive this treatment. JA clones were subcloned in medium without selecting stimulus and have remained resistant throughout prolonged culture periods. The expression level of CD95 is within the wild-type range, and its cytoplasmic tail is wild-type according to nucleotide sequencing. The cross-resistance suggested that CD95 and the DNA-damaging stimuli mediate apoptosis. These clones proved cross-resistant to apoptotic cell death, whereas this does not occur in JA1.2 resistant to total radioactivity in phosphatidylserine and phosphatidylethanolamine, which remained unaltered upon stimulation.

RESULTS

A Common Aspect in Apoptosis Signaling Induced by CD95, Etoposide, and γ-Radiation—The human T-acute lymphoblastic leukemia cell line Jurkat is sensitive to multiple apoptotic stimuli, including CD95 triggering, exposure to certain anticancer drugs, and γ-radiation (IR). We have selected variant clones from the wild-type Jurkat line for resistance to CD95-mediated apoptosis. These clones proved cross-resistant to apoptosis induction by etoposide and IR, as shown in Fig. 1A. Whereas the wild-type clone J16 undergoes dose- and time-dependent apoptosis in response to anti-CD95 mAb, etoposide, or IR, apoptosis incidence in the variant clone JA1.2 does not exceed background levels, even at high dose stimulation. Yet, the resistant JA1.2 variant cells sense the DNA damage as induced by etoposide and γ-radiation since they arrest in the G2 phase of the cell cycle upon treatment, as wild-type Jurkat cells do. However, in wild-type cells, the G2 arrest is followed rapidly by apoptotic cell death, whereas this does not occur in JA1.2 cells (Fig. 1B).

Apoptosis Signaling Induced by Etoposide and γ-Radiation Does Not Require Ded-Containing Caspases—The cross-resistance suggested that CD95 and the DNA-damaging stimuli etoposide and γ-radiation require common molecular events to induce apoptosis. To find a possible point of convergence in apoptosis signaling induced by these three inputs, we first tested whether the anticancer treatments induced caspase-8

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processing, as does CD95 (15). Immunoblotting of whole cell lysates with an antiserum directed against the amino terminus of caspase-8 allowed detection of two proforms, of ~54 and 50 kDa, prior to stimulation. CD95 stimulation gave rise to a caspase-8 doublet at ~40 and 36 kDa within ~1 h, which increased in intensity in the following hours, whereas the pro-caspase-8 signal decreased concomitantly (Fig. 2). This is consistent with the release of the carboxyl-terminal caspase fragment (15). In addition, a 23-kDa species was detected, which most likely represents the amino-terminal region of caspase-8 after release of the second caspase subunit. Etoposide and IR also induced caspase-8 processing, but to a minor extent. In these cases, only the 40/36-kDa digestion products could be detected, even upon long-term stimulation (Fig. 2). All three stimuli failed to induce caspase-8 processing in the resistant clone JA1.2 (Fig. 2).

To determine whether caspase-8 is instrumental in apoptosis induction by etoposide and IR, we employed Jurkat cells stably transfected with FLIP<sub>L</sub>cDNA. It was previously demonstrated that apoptosis induction by CD95 and TRAIL receptors is inhibited in these transfectants (9). Fig. 2 shows that in these JFL2 cells, the dramatic caspase-8 processing induced by CD95 was blocked. Also, the minor caspase-8 processing induced by etoposide and IR was no longer observed. Whereas FLIP<sub>L</sub>-inhibited CD95-induced apoptosis, it did not affect the apoptotic response to etoposide and IR (Fig. 3). Apparently, activation of FLIP<sub>L</sub>-inhibitable, DED-containing caspases is required for apoptosis induction by CD95, but not for apoptosis induction by etoposide or IR.

**Apoptosis Signaling Pathways Induced by CD95, Etoposide, and γ-Radiation Are under Common Mitochondrial Control—**

Since apoptosis signaling induced by CD95, etoposide, and γ-radiation did not converge at the level of DED-containing caspases, we investigated whether the presumed mitochondrial caspase activation complex might be involved in these three pathways. To directly examine mitochondrial involvement, release of cytochrome c into the cytoplasm was determined. Fig. 4 shows that CD95, etoposide, and IR induced release of cytochrome c in control Jurkat cells. CD95-induced cytochrome c release was inhibited by FLIP<sub>L</sub> overexpression, indicating that...
CD95 communicates to the mitochondria via caspase-8. FLIP_L did not inhibit cytochrome c release in response to etoposide or IR, substantiating the lack of caspase-8 involvement in the DNA damage pathways (Fig. 4).

Cytochrome c release in response to all three stimuli was severely reduced in the resistant JA1.2 clone (Fig. 4), suggesting that the apoptosis signaling pathways induced by CD95, etoposide, and IR all depend on a contribution by the mitochondria. Generally, a possible contribution of the mitochondrial pathway to apoptosis induction is assessed by the effects of Bcl-2 family members on the apoptotic response. Whereas apoptosis induction by DNA-damaging regimens such as etoposide treatment and IR is consistently modulated by Bcl-2 family members (25, 26), it has been unclear whether death receptor signaling is similarly regulated. For instance, in transgenic thymocytes, CD95-mediated apoptosis is not affected by Bcl-2 or Bax overexpression (26, 48), whereas in certain cell lines, Bcl-2 and Bcl-xL were found to inhibit tumor necrosis factor receptor-1- and/or CD95-induced apoptosis (45, 49, 50). Recently, it was suggested that in certain cells (type I), CD95 employs a Bcl-2-independent pathway, whereas in other cells (type II), including Jurkat, Bcl-2 and Bcl-xL inhibit CD95 signaling (51). Using the previously described Bcl-2-overexpressing Jurkat cells (45, 50), we also found that not only etoposide- and IR-induced cytochrome c release and apoptosis were inhibited, but also CD95-induced cytochrome c release (Fig. 4) and apoptosis (Fig. 5). The combined data suggest a point of convergence in apoptosis signaling induced by CD95, etoposide, and IR at the mitochondria.

Surprisingly, caspase-8 processing induced by CD95 was inhibited by Bcl-2 overexpression (Fig. 2). This has also been observed by Scaffidi et al. (50), who suggested that CD95 signaling in Jurkat cells involves rapid, low level caspase-8 activation at the DISC, followed by a slow, more pronounced response, which is under mitochondrial control. Rapid caspase-8 activation within minutes could not be observed in total lysates of Jurkat cells (data not shown) (50). Since FLIP_L inhibits caspase-8 activation at the DISC (9), we cannot assess whether the late, Bcl-2-controlled caspase-8 response contributes to apoptosis induction by CD95. Bcl-2 also inhibited caspase-8 processing as induced by etoposide and IR (Fig. 2).

Regulation of Effector Caspase Processing in Response to CD95, Etoposide, and γ-Radiation—To further delineate the convergence in apoptosis signaling, we set out to identify effector caspases that were under the control of CD95, etoposide, and IR. CD95 effectively induced caspase-3, -6, and -7 processing in control Jurkat cells (Fig. 6A). In response to etoposide treatment and IR, caspase-3 and caspase-6 were cleaved to a minor degree, whereas caspase-7 processing was readily detectable (Fig. 6). FLIP_L inhibited CD95-induced caspase-3, -6, and -7 processing (Fig. 6B), indicating that a DED-containing caspase (most likely caspase-8) is upstream from these effector caspases in the CD95 pathway. In contrast, effector caspase cleavage in response to etoposide and IR occurred independent of caspase-8 activation (Fig. 6B). CD95-, etoposide-, and IR-induced effector caspase processing did not occur in the resistant JA1.2 clone or in Bcl-2-overexpressing Jurkat cells (Fig. 6B). We conclude that CD95 and the DNA-damaging regimens can signal to the same effector caspases, but with different efficiencies. CD95 signals to effector caspases via caspase-8, whereas etoposide and IR use a caspase-8-independent route. Downstream from caspase-8, CD95-, etoposide-, and IR-in-
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**DISCUSSION**

Our finding that cells selected for resistance to CD95-induced apoptosis were cross-resistant to apoptosis induction by etoposide and IR suggested that apoptosis signaling induced by these three stimuli has a common aspect. Since CD95 signaling involves FADD-mediated recruitment of caspase-8 and its subsequent proteolytic activation, we examined whether etoposide and IR also induced caspase-8 processing. Caspase-8 was cleaved to a minor extent in etoposide-treated and irradiated cells, but this proved to be dispensable for effector caspase processing and the cell death response. This is an important finding since it was suggested previously that anticancer drugs (13, 14) and γ-radiation (51) can induce apoptosis by activating the CD95 receptor system. Anticancer drugs would do so by inducing synthesis of CD95 ligand (13, 14) and, in certain cases, also the receptor (14). CD95 was implicated in apoptosis induction by γ-radiation based on diminished radiation sensitivity of CD95-deficient splenocytes from lpr mice (51).

Such a mechanism would obviously account for the observed cross-resistance. However, involvement of the CD95 receptor-
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![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 7. Ceramide and apoptosis signaling induced by CD95, etoposide, and IR. A, J16 wild-type cells and the JA1.2 clone were treated with anti-CD95 mAb CH-11 at 500 ng/ml, etoposide at 5 μg/ml, or IR at 15 Gy, and ceramide (Cer) levels were determined at the indicated time points and are expressed as -fold increase relative to levels were determined at the indicated time points. B, empty vector-transfected (J neo) and bcl-2 cDNA-transfected (J Bcl-2) Jurkat cells and JA1.2 were treated with vehicle (dimethyl sulfoxide [DMSO]) or 75 μM C<sub>C</sub>-ceramide (C<sub>C</sub>-Cer) for the indicated time periods. Proteolytic processing of caspase-6 was determined by immunoblotting for various Bcl-2 family members.**

**REFERENCES**


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