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Werner, A.B.

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Submitted
Truncated Bid signals apoptosis in response to DNA damage

Requirement for aspartate-cleaved Bid in apoptosis signaling by DNA damaging anti-cancer regimens*

Arlette B. Werner, Stephen W.G. Tait, Evert de Vries, Eric Eldering⁵, and Jannie Borst

Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam;⁵Department of Experimental Immunology, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands

*This work was supported by grants from the Dutch Cancer Foundation and by a Marie Curie fellowship from the European Community to S.W.G.T. ¹The abbreviations used are: Cyt c, cytochrome c; GFP, enhanced green fluorescent protein; mAb, monoclonal antibody; MEF, mouse embryonic fibroblasts; MIB, mitochondrion incubation buffer; MLPA, multiplex ligation-dependent probe amplification; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; SDS, sodium dodecyl sulfate; si, short interfering.

SUMMARY

Lymphoid malignancies can escape from DNA damaging anti-cancer drugs and γ radiation by blocking apoptosis signaling pathways. How these regimens induce apoptosis is incompletely defined, especially in cells with non-functional p53. We report here that the BH3-only Bcl-2 family member Bid is required for mitochondrial permeabilization and apoptosis-induction by etoposide and γ radiation in p53 mutant T leukemic cells. Bid is not transcriptionally upregulated in response to these stimuli, but activated by cleavage on aspartate residues 60 and/or 75, which are the targets of Caspase-8 and Granzyme B. Bid activity is not inhibitable by c-FlipL or dominant negative Caspase-9 and therefore independent of inducer caspase activation by death receptors or the mitochondria. Caspase-2, which has been implicated as inducer caspase in DNA damage pathways, appeared to be processed in response to etoposide and γ radiation, but downstream of Caspase-9. Knock down of Caspase-2 by short interfering RNA further excluded its role in Bid activation by DNA damage. Caspase-2 was implicated in the death receptor pathway though, where it contributed to effector caspase processing downstream of inducer caspases. Serpins SPI-6 or PI-9 could not block the DNA damage pathway, excluding a role for Granzyme B in the generation of active Bid. We conclude that Bid, cleaved by an undefined aspartate-specific protease, can be a key mediator of the apoptotic response to DNA damaging anti-cancer regimens.
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INTRODUCTION

Execution of apoptosis requires the activation of effector caspases, which can be accomplished via the death receptor- or the mitochondrial pathway. Death receptor-induced apoptosis does not require de novo protein synthesis, but the mere recruitment of preformed molecules to the receptor, including the inducer Caspases-8 and/or -10. Most cellular stresses, however, rely on the primordial mitochondrial pathway, which requires more elaborate intracellular events to become activated. Depending on the stimulus, its activation may involve new synthesis of apoptosis signaling molecules, and/or their post-translational modification. The ultimate induction of mitochondrial permeability in this pathway allows the release of Cytochrome c (Cyt c), Smac/Diablo and Omi/HtrA2, which are essential for activation of the inducer Caspase-9.

The response of the mitochondrion to upstream stimuli is a critical control point in apoptosis signaling. It is crucial, therefore, to understand how death signals are transmitted to this organelle. In mammals, multiple pro-apoptotic BH3-only members of the Bcl-2 family are specialized in sensing partially distinct stimuli. They integrate apoptotic stimuli into one single pathway by triggering mitochondrial permeabilization by the Bcl-2 family members Bax and/or Bak. There are two prominent models for mode of the action of BH3-only proteins. In one, they transiently interact with Bax and/or Bak, inducing them to form pores in the mitochondrial membrane. In this scenario, inhibitory Bcl-2 family members sequester BH3-only proteins to prevent their pro-apoptotic action. In the other model, BH3-only proteins interact with inhibitory Bcl-2 family members and thereby neutralize their function, which results in Bax/Bak activation.

To prevent inappropriate cell death, BH3-only family members are kept in check by mechanisms, which are tailored to each individual member. For example, Noxa and Puma are under transcriptional control, in particular by p53 in the DNA damage response. Other BH3-only proteins are regulated by various modes of posttranslational modification. Bim and Bmf are sequestered to the cytoskeleton and released upon phosphorylation. Bad is sequestered in the cytosol and released upon dephosphorylation. Bid requires proteolytic cleavage to produce an active carboxy-terminal fragment, which is achieved by Caspase-8 in the death receptor pathway and by Granzyme B in apoptosis triggered by cytolytic T cells.

It is important to elucidate the molecular events involved in apoptosis signaling by anti-cancer therapies. The required active participation of cancer cells in bringing about their own demise implies that they can also put up resistance. Whether resistance to apoptosis generally confers therapy resistance is debated, since cells may well die a
non-apoptotic death or be irreversibly arrested in cell cycle in response to anti-cancer regimens\textsuperscript{12}. However, for lymphoid malignancies, convincing evidence has been provided that loss of p53 or Bcl-2 overexpression and resulting apoptosis resistance promotes therapy resistance in vivo\textsuperscript{13,14}.

Although \( \gamma \) radiation and DNA damaging anti-cancer drugs are complex inputs, there is a great deal of molecular evidence that the apoptosis signaling pathways initiated by these stimuli emerge from the DNA damage response. DNA damage is sensed by ATM and Chk2 kinases, which phosphorylate and stabilize p53, allowing it to function as a transcription factor\textsuperscript{15,16}. In this way and possibly also via transcription-independent mechanisms, p53 can bring about a cell cycle arrest and apoptosis\textsuperscript{17}. Cell cycle arrest usually precedes apoptosis, to allow for repair of DNA damage. Whether apoptosis ensues is negotiated between the DNA repair machinery and the apoptotic machinery\textsuperscript{18}. p53 mediates transcription of pro-apoptotic Bcl-2 family members, such as Bax\textsuperscript{19}, Puma\textsuperscript{6}, Noxa\textsuperscript{7} and Bid\textsuperscript{20}, of death receptors like CD95\textsuperscript{21} and TRAIL receptor-2\textsuperscript{22}, of the Caspase-9 activator Apaf-1\textsuperscript{23} and of HtrA2/Omi\textsuperscript{24}. In this scenario, one can envision that cells are sensitized for apoptosis induction via the mitochondrial pathway, as well as the death receptor pathway. Whether they undergo apoptosis may depend on expression levels of pro-apoptotic Bcl-2 family members and/or availability of death receptor ligand.

Wild-type p53 is not absolutely required for apoptosis-induction by DNA damaging regimens, as shown in cycling peripheral T lymphocytes and lymphoma cells of p53\textsuperscript{-/-} mice\textsuperscript{25} and in various p53 mutant cancer cell lines. For instance, the Jurkat T leukemic cells used in this study are highly sensitive to DNA damaging anti-cancer drugs, such as the topoisomerase II inhibitor etoposide, as well as to \( \gamma \) radiation\textsuperscript{26,27}, while they harbor mutant p53, which is transcriptionally inactive\textsuperscript{28,29}. In cycling murine T lymphocytes and lymphoma cells\textsuperscript{25}, as well as in Jurkat cells\textsuperscript{26,27}, Bcl-2 overexpression inhibits apoptosis-induction by DNA damaging anti-cancer drugs and \( \gamma \) radiation, indicating that in these cells the p53-independent DNA damage response relies on the mitochondrial pathway for caspase activation. However, which signaling molecules activate the mitochondria in this pathway is unclear.

In the work reported here, we have addressed this question and found that Bid is the key mediator of mitochondrial activation in the p53-independent DNA damage response in Jurkat T cells. We provide evidence that Bid needs to be proteolytically activated by an aspartate-specific protease to perform its function in this pathway. However, we have excluded Granzyme B and all known inducer caspases from playing a role in Bid processing. This includes Caspase-2, which has been featured recently as
an activator of the mitochondrial pathway in response to DNA damaging anti-cancer drugs\textsuperscript{30,31}.

**EXPERIMENTAL PROCEDURES**

**Reagents** - Mouse anti-human CD95 monoclonal antibody (mAb) 7C11 was obtained from Immunotech (Marseille, France), soluble human recombinant TRAIL and enhancer from Alexis (Laufelfingen, Switzerland) and etoposide from Sigma. Polyclonal rabbit anti-Bid antibody was raised in our laboratory against a fusion protein of glutathione S-transferase and full-length Bid. It detects full-length and truncated (t)Bid\textsuperscript{32}. Anti-actin mAb C4 was obtained from Roche Molecular Biochemicals, anti-Cyt c mAb 7H8.2C12, anti-Caspase-2/ICH-1, mAb (clone 35) and anti-active Caspase-3 mAb were from BD Biosciences. Anti-HA mAb 12CA5 and anti-VSV mAb P5D4 were used as purified Ig prepared from available hybridomas. Rabbit anti-Caspase-9 polyclonal antibody was purchased from New England Biolabs. Horseradish peroxidase-conjugated rabbit anti-mouse Ig and swine anti-rabbit Ig were obtained from DAKO A/S (Glostrup, Denmark). Protein A-Sepharose beads and the enhanced chemiluminescence (ECL) kit were purchased from Amersham Biosciences.

**Cells and stimulation** - The J16 clone was derived from the human T-acute lymphoblastic leukemia cell line Jurkat by limiting dilution and selected for CD95 sensitivity\textsuperscript{26,37,32}. CD95-resistant JA variant clones were derived by limiting dilution from the parental Jurkat line after 5 weeks of selection for cells resistant to anti-CD95 mAb\textsuperscript{26}. The functional status of p53 was analyzed in J16 and JA clones by monitoring the transactivation activity of separated alleles in yeast according to Ishioka et al.\textsuperscript{44}. All cells expressed one functional and one non-functional allele. The JS/J mixed lymphocyte culture was generated by coculturing peripheral blood mononuclear cells of donor JS with irradiated Jurkat cells. Allogeneic responder T cells were propagated by weekly stimulation with feeder cell mixture and cloned by limiting dilution at 1 cell/well in the same feeder mixture\textsuperscript{35}. T cell clones JS/I7 and JS/I29 were selected for cytotoxicity towards J16 cells as displayed in a conventional \textsuperscript{51}Cr release assay. Jurkat cells were cultured in Iscove's modified Dulbecco's medium and Phoenix-Ampho cells in Dulbecco's modified medium, both supplemented with 8% fetal calf serum, 2 mM glutamine and antibiotics. Prior to stimulation, Jurkat clones were suspended in serum-free Yssel's medium\textsuperscript{26} and seeded at 1 x 10\textsuperscript{6} per ml, 200 µl per well in round-bottom 96-well plates for apoptosis assays and at 5-10 x 10\textsuperscript{5} per ml in 24-well culture plates for Cyt c release assays. Prior to irradiation or treatment with etoposide, cells were cultured overnight in Yssel's medium. After addition of stimulus, cells were incubated for the indicated time periods at 37°C, 5% CO\textsubscript{2}. Routinely, cells were stimulated by incubation with 50 ng/ml anti-CD95 mAb for 4-6 h, with 100 ng/ml recombinant TRAIL plus ten-fold excess of enhancer for 6-8 h, or with 10 µg/ml etoposide for 16-18 h. Cells were irradiated with 30 Gy using a \textsuperscript{153}Cs source (415 Ci; Von Gahlen Nederland, B.V.) and incubated for 16-18 h.

**Apoptosis assays** - To measure nuclear fragmentation (subdiploid DNA content), cells were lysed in 0.1% Triton X-100, 0.1% sodium citrate, 50 µg/ml propidium iodide (PI) as
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Described\(^\text{16}\). Fluorescence intensity of PI-stained nuclei was determined on a FACScan (Becton Dickinson, San Jose, CA) and data were analyzed using CellQuest software. Levels of active Caspase-3 were determined as follows: After stimulation, cells were fixed in 4% paraformaldehyde, washed in phosphate-buffered saline (PBS) with 0.1% saponin and 0.5% bovine serum albumin (BSA) and stained for 45 min on ice with phycoerythrin-conjugated anti-active Caspase-3 mAb in PBS with 0.1% saponin and 0.5% BSA. Subsequently, cells were washed and analyzed by flow cytometry. For determination of apoptosis inflicted by the cytotoxic JS/J clones, effector T cells and J16 target cells were cocultured in 24 well plates at the indicated ratios and subsequently washed in PBS, fixed for 2 h at -20°C with 70% ethanol, washed in PBS, incubated for 1 h at 4°C with 100 μg/ml RNase and 20 μg/ml PI and analyzed by flow cytometry on a FACScan. Percentage of subdiploid PI-positive particles was scored as percentage apoptosis. Background apoptosis in effector and target cells that had been cultured alone was minimal, but subtracted from the percentage apoptosis determined in all cocultures.

**Immunoblotting** - For monitoring Cyt c release, stimulated cells were washed twice with ice-cold PBS, suspended in 100 µl extraction buffer (50 mM PIPES-KOH pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl\(_2\), 1 mM dithiothreitol and protease inhibitors) and allowed to swell on ice for 30 min. Cells were homogenized by passing the suspension through a 25-gauge needle (10 strokes). Homogenates were centrifuged in a Beckman Airfuge at 100000 g for 15 min at 4°C and supernatants were harvested as cytosolic fractions. Fifteen μg of cytosolic protein, as determined by the Bio-Rad protein assay (Bio-Rad Laboratories, München) were loaded per lane onto 15% sodium dodecylsulfate (SDS)-polyacrylamide gels and blotted onto nitrocellulose. For monitoring protein expression levels or caspase processing, cells were washed with PBS and lysed in 10 mM Triethanolamine-HCl pH 7.6, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA and protease inhibitors. Lysates were cleared by centrifugation and analyzed for protein content by Bio-Rad protein assay. Equal amounts of protein (50-75 μg per lane) were separated on 12% SDS-polyacrylamide gels and transferred to nitrocellulose sheets. After transfer, membranes were blocked for 1 h in PBS, 0.05% Tween with 5% not-fat dry milk and probed in PBS, 0.05% Tween with anti-Cyt c mAb (1:1000) and anti-actin mAb (1:10000), or with anti-Caspase-2 (1:1000), anti-Bid (1:1000), or anti-Caspase-9 antibodies (1:2000). After incubation with a 1:7500 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody, immunostained proteins were visualized by ECL.

**Isolation of mitochondria** - Mouse liver cells were lysed by Dounce homogenization in mitochondrial incubation buffer (MIB): 5 mM HEPES-KOH pH 7.2, 250 mM mannitol, 0.5 mM EGTA, 0.1% (w/v) BSA, 1 μg/ml leupeptin and 0.1 mM phenylmethylsulfonyl fluoride. Nuclei and debris were removed by centrifugation at 600 g for 5 min at 4°C and a pellet containing mitochondria was obtained by a spin at 10000 g for 10 min at 4°C. The pellet was suspended in MIB and layered on a gradient consisting of layers of 10%, 18%, 30% and 70% Percoll in 25 mM HEPES-KOH pH 7.2, 225 mM mannitol, 0.5 mM EGTA and 0.1% (w/v) BSA. Purified mitochondria were collected at the 30%/70% Percoll interphase after centrifugation in a SW-41 rotor for 35 min at 13500 g at 4°C. The harvested fraction was at least 5-fold diluted in MIB and centrifuged for 10 min at 6300 g at 4°C. After two more washes in MIB, mitochondria were suspended in Wang buffer B, which is 20 mM HEPES-KOH pH 7.5, 220 mM mannitol, 68 mM sucrose, 100 mM KCl, 1.5 mM MgCl\(_2\), 1 mM
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EDTA, 1 mM EGTA, 1 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride to a protein concentration of 5 mg/ml.

In vitro mitochondrion assay - Cytosols were prepared by hypotonic lysis as described above in Wang buffer B with 10 mM KCl and subsequently incubated with anti-Bid or normal rabbit serum and protein A-Sepharose beads five times for 1 h at 4°C. Purified mitochondria (25 µg/sample) were incubated with 100 µg cytosolic protein in a final volume of 30 µl of Wang buffer B at 30°C for 1 h and subsequently centrifuged for 5 min at 10000 g at 4°C. Mitochondrial pellets were solubilized in SDS sample buffer and separated by 15% SDS-polyacrylamide gel electrophoresis at 12.5 µg protein/sample. Immunoblotting for Cyt c content was performed as described above.

Retroviral gene transduction - cDNAs encoding Bcl-2, BidD60E/D75E, dominant negative (dn) FADD, c-FlipL, the dn active site mutant C287A of Caspase-9 and carboxy-terminally HA-tagged PI-9 (a gift from Dr. J.P. Medema, Leiden University Medical Center, Leiden, The Netherlands) were cloned into LZRS-IRES-Zeo/pBR (Bcl-2) or LZRS-IRES-GFP (other cDNA), both derivatives of LZRS-pBMN-lacZ, which was provided by Dr. G. Nolan (Stanford University School of Medicine, San Francisco, CA). LZRS-IRES-GFP with carboxy-terminally VSV-tagged SPI-6 was provided by J.P. Medema. Retrovirus production and transduction were carried out as previously described. Transduced cells were selected for enhanced green fluorescent protein (GFP) expression using a MoFlo high-speed cell sorter (Cytomation, Fort Collins, CO).

Stable knock down of human Caspase-2 by short interfering (si)RNA - pSUPER-GFP (a gift from Dr. H. Spits and R. Schotte, Amsterdam Medical Center, Amsterdam The Netherlands) is identical to the previously described pSUPER vector, except that it expresses GFP under a phosphoglycerate kinase promoter. Complementary sense and anti-sense oligonucleotides (Caspase-2 si II sense oligo 5' acagctgtgttgagca 3'; Caspase-2 si III sense oligo 5' tgcaagagaaactgcaga 3') were annealed and cloned into pSUPER-GFP. The siRNA GFP cassette was cloned into pSin, an LZRS-based retroviral vector that allows expression of the siRNA under a Pol III promoter (kindly provided by Dr. H. Spits and R. Schotte). Packaging cell supernatants containing ecotropic retrovirus encoding Caspase-2 si II and Caspase-2 si III were used to transduce J16 cells expressing the ecotropic retroviral receptor. (This receptor was introduced by stable transduction of the cDNA cloned in pBabe-puro and selection by puromycin). Transduced cells expressing Caspase-2 si constructs were selected for GFP expression using a MoFlo high-speed cell sorter.

Multiplex ligation-dependent probe amplification (MLPA) - The principle of MLPA has been described. This method allows amplification and quantitative analysis of transcripts from multiple genes in a one-tube assay. Total RNA was prepared from non-stimulated or 3, 6 or 9 h etoposide-stimulated or γ-irradiated J16 cells by the Trizol method and reverse transcribed using a gene-specific oligonucleotide probe mix. The resulting cDNA was annealed overnight to the MLPA probe mix. Probes used in this study were designed to hybridize with cDNA of 36 human apoptosis related genes. Detailed reaction conditions for MLPA with these particular probes and their performance have been described. The annealed oligonucleotides and cDNAs were ligated and products were amplified by polymerase chain reaction (PCR), with one unlabeled and one fluorescently labeled primer.
The resulting DNA mixture was analyzed on an Applied Biosystems 3100 capillary sequencer with GeneScan and Genotyper software packages (Applied Biosystems, Warrington, UK). As expression of housekeeping genes varied minimally throughout the time courses, the sum of the fluorescence intensities of all products amplified in one reaction was set at 100% to correct for fluctuations in total signal between samples and individual peaks were calculated relative to the 100% value.

RESULTS

Common signaling requirements for apoptosis-induction by DNA damaging anti-cancer regimens and death receptors

In wild-type J16 Jurkat T cells, apoptosis-induction by the topoisomerase II inhibitor etoposide and γ radiation shares aspects with apoptosis-induction by the death receptor CD95. This was first discovered when variant Jurkat cells, selected for resistance to CD95-mediated apoptosis, proved cross-resistant to these DNA damaging anti-cancer regimens. In variant JA cells, etoposide and γ radiation bring about a cell cycle arrest in G2, as they do in wild-type J16 cells (Fig. 1A). However, while this arrest is followed by an apoptotic response in case of J16, JA cells do not undergo apoptosis. Upon prolonged incubation, they remain arrested in G2 and ultimately die by necrosis within a period of a week (results not shown). Apparently, these cells have retained the capacity to sense the detrimental consequences of treatment with etoposide and γ radiation and can translate these in a cell cycle arrest, but have lost the ability to activate the apoptotic machinery.

It has been reported previously that in Jurkat T cells, DNA damaging anti-cancer drugs and γ radiation induce apoptosis in a death receptor-independent manner. This was confirmed in this study by retroviral transduction of J16 cells with Δn FADD and c-FlipL. Δn FADD, which has a death domain but lacks the death effector domain, abrogates death receptor signaling by disallowing recruitment of Caspase-8/10 to the death receptor. C-FlipL blocks activation of Caspase-8/10 by displacing it from FADD at the cytoplasmic tail of death receptors. While Δn FADD and c-FlipL effectively blocked CD95-induced apoptosis, they did not interfere with apoptosis-induction by etoposide and γ radiation (Fig. 1B), indicating that these regimens do not require death receptor engagement and/or FADD and Caspase-8/10 function to convey the apoptotic signal.

An aspect of apoptosis signaling that is shared by DNA damaging anti-cancer regimens and death receptors in Jurkat T cells is the requirement of a mitochondrial contribution to activation of the apoptotic execution machinery. This is shown for J16 cells in Fig. 1C, in which retrovirus-mediated overexpression of Bcl-2 not only blocks
the apoptotic response to etoposide and γ radiation, but also strongly inhibits apoptosis-induction via CD95 and TRAIL receptor. In JA cells, the capacity of etoposide, γ radiation and CD95 to bring about Cyt c release is abrogated, confirming that the response to these stimuli is under common mitochondrial control. We conclude that etoposide and γ radiation induce apoptosis in J16 cells via a mitochondrial pathway that shares aspects with the death receptor pathway downstream from Caspase-8/10.

**Figure 1**- Apoptosis signaling by DNA damaging inputs in Jurkat cells shares aspects with death receptor signaling, but is independent of death receptor activation. A, The wild-type clone J16 and the variant clone JA1.2, which was selected for CD95-resistance, were stimulated with anti-CD95 mAb or etoposide (E), or γ-irradiated (IR). The medium control (M) was incubated under the same conditions. Apoptosis was read out as nuclear fragmentation by PI staining of nuclei and FACS analysis. B, J16 cells, stably transduced with empty vector, c-FlipL or dn FADD cDNA were treated with etoposide, γ radiation or anti-CD95 mAb and apoptosis was read out as indicated in A. C, J16 cells transduced with empty vector or Bcl-2 cDNA were treated with etoposide, γ radiation or anti-CD95 mAb and apoptosis was read out as indicated in A. All data are representative of multiple independent experiments and show means ± S.D. from triplicate samples in one experiment.

**Etoposide and γ radiation convey the apoptotic signal via presynthesized, aspartate-cleaved Bid**

To identify the BH3-only protein that may be instrumental in the DNA damage pathway in Jurkat cells, we first determined which apoptosis-regulatory proteins were transcriptionally regulated by etoposide and γ radiation. J16 cells were stimulated for 3, 6 and 9 h, a time frame in which Cyt c release increments to maximal levels, and RNA was isolated. The transcript levels of most known BH3-only proteins, Bak, Bax, inhibitory Bcl-2 family members and some other apoptosis regulators were determined by MLPA, a PCR-based assay that allows the quantitative comparison of multiple transcripts amplified in one reaction. No or only marginal up- or downregulation was observed for Bcl-2 family members, including Bid, Noxa, Bax, Bak, Bcl-2 and Bcl-
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xL (Supplemental Fig.). The exception was Puma, for which transcripts were not detectable in unstimulated cells, but modestly upregulated in stimulated cells. However, expression of Puma siRNA construct, which had been validated in transiently transfected HeLa and 293T cells, had no effect on apoptosis-induction by etoposide or γ radiation (results not shown). Therefore, analysis of new transcription in response to DNA damage did not clarify which BH3-only protein(s) may convey the apoptotic signal in the pathways activated by etoposide and γ radiation in Jurkat cells.

Figuur 2 - Aspartate-cleaved Bid is required for Cyt c release and apoptosis in response to etoposide and γ radiation. A, In vitro mitochondrion assay. Mitochondrion-free cytosols were derived from J16 cells, which had been incubated without stimulus (medium) or treated with etoposide (E) or γ radiation (IR) for the indicated time periods. Cytosols were immunodepleted for Bid (ΔBid) or mock-depleted with normal rabbit serum (control) and incubated with mouse liver mitochondria for 60 min at 30°C. Cyt c content of the mitochondrial fraction was assayed by immunoblotting. B, J16 cells were transduced with empty vector or the Bid D60E/D75E mutant cDNA, in which Caspase-8 and Granzyme B cleavage sites were mutated. Cells were stimulated with the indicated concentrations of etoposide or doses of γ radiation. Apoptosis was read out as nuclear fragmentation. Data represent means ± S.D. of duplicate samples from three independent experiments. C, In vivo Cyt c release assay. The same cells as described for B were stimulated with etoposide (E) or γ radiation (IR), or left untreated (M) for the indicated time periods. Mitochondrion-free cytosols were prepared and assayed for the presence of Cyt c and actin (as a loading control).
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We have demonstrated previously that in JA cells, tBid cannot signal to mitochondria. Recombinant tBid, when incubated with exogenous mouse liver mitochondria in the presence of cytosol from JA cells was unable to bring about Cyt c release, while it functioned normally in the context of J16 cytosol. Therefore, common resistance to CD95-, etoposide and γ radiation-induced apoptosis in JA cells can possibly be attributed to the inability of tBid to induce mitochondrial permeabilization in all these pathways. To explore whether Bid might be the common mediator involved in signaling to the mitochondria in the DNA damage and death receptor pathways, we first used an *in vitro* mitochondrial assay. Cytosols were derived from wild-type J16 cells, which had been stimulated with etoposide or γ radiation or left untreated for various periods of time. These *in vivo* activated cytosols were either mock depleted or depleted of Bid by repeated immunoprecipitation and incubated with mouse liver mitochondria. Subsequently, mitochondria were assayed for the presence of Cyt c. In case mitochondria were incubated with mock-depleted etoposide- or radiation-activated cytosols, Cyt c was effectively released (Fig. 2A). However, etoposide- and radiation-activated cytosols depleted of Bid lost the capacity to release Cyt c from mouse liver mitochondria, indicating that Bid is required to permeabilize mitochondria in response to these stimuli.

In the death receptor pathway, Bid needs to be cleaved to become active and convey the apoptotic signal to mitochondria. Therefore, we investigated whether overexpression of a non-cleavable Bid mutant could inhibit the apoptotic response to DNA damage. J16 cells were retrovirally transduced with a Bid cDNA, containing point mutations that alter the Caspase-8/10 (D60E) and Granzyme B (D75E) cleavage sites by substituting aspartate for glutamate. This non-cleavable Bid mutant has previously been shown to block signaling to the mitochondria by CD95 and TRAIL receptor in J16 cells. We demonstrate here that it also fully inhibits etoposide- and γ radiation-induced apoptosis in these cells (Fig. 2B). Immunoblotting of mitochondrion-free cytosols, derived from J16 cells treated with etoposide or γ radiation, showed that the BidD60E/D75E mutant impeded Cyt c release in both cases (Fig. 2C). We conclude that, surprisingly, the DNA damaging anti-cancer regimens etoposide and γ radiation require aspartate-cleaved Bid to induce Cyt c release and apoptosis in Jurkat T cells.

### Bid acts upstream of the mitochondria in response to DNA damage

It is known that Caspase-3 can cleave Bid at the same aspartate residue as Caspase-8/10 and with that amplifies Cyt c release in a mitochondrial feedback loop. Therefore, we assessed whether in the DNA damage pathway cleaved Bid was required to convey the initial apoptotic signal to the mitochondria, or acted downstream of the mitochondria in
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To this end, J16 cells were retrovirally transduced to express an active site mutant of Caspase-9, which blocks the apoptotic pathway directly downstream of the mitochondria. This dn Caspase-9 strongly inhibited the apoptotic response to etoposide and γ radiation, as expected from the complete reliance on the mitochondrial pathway for effector caspase activation (Fig. 3A). Immunoblotting of mitochondrion-free cytosols, prepared from J16 cells stimulated with etoposide or γ radiation, showed that Cyt c release was not detectably reduced by expression of dn Caspase-9 (Fig. 3B). From this, it can be concluded that the Cyt c release observed in response to etoposide and γ radiation does not rely on a Caspase-9-dependent feedback loop. Since overexpression of the Bid mutant completely prevented the release of Cyt c (Fig. 2C), we can conclude that cleaved Bid is an obligatory signaling intermediate in the DNA damage pathway upstream of the mitochondria.

**Figure 3** - Cyt c release induced by etoposide and γ radiation does not involve a Caspase-9-dependent feedback loop. A, J16 cells were transduced with empty vector or a vector encoding dn Caspase-9. Cells were treated with the indicated concentrations of etoposide or doses of γ radiation. Apoptosis was read out as nuclear fragmentation. Data represent means ± S.D. derived of duplicate samples from three independent experiments. B, In vivo Cyt c release assay. J16 cells transduced as described in A, were treated for the indicated time periods with etoposide (E), γ radiation (IR) or left untreated (medium). Mitochondrion-free cytosols were assayed for the presence of Cyt c and actin by immunoblotting.

Caspase-2 processing in response to DNA damage and death receptor triggering occurs downstream of initiator proteases

Next, we investigated which protease cleaves Bid in the DNA damage pathway. Apart from Caspase-9, we could rule out Caspases-8 and -10, since J16 cells overexpressing c-FlipL were not affected in their apoptotic response to treatment with etoposide and γ radiation (Fig. 1B). Moreover, we had demonstrated previously that the pan-caspase inhibitor zVAD-fmk did not block Cyt c release induced by these stimuli in J16 cells. Recently, it was found that in certain cell types Caspase-2 participates upstream of the
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mitochondria in the response to DNA damaging anti-cancer drugs. Since Caspase-2 cannot effectively be inhibited by zVAD-fmk, it was a good candidate to be involved in Bid processing. Therefore, we first determined whether Caspase-2 was processed in response to etoposide and γ radiation and whether this occurred upstream of Bid. J16 cells overexpressing the Bid D60E/D75E mutant and vector control cells were stimulated with etoposide, γ radiation, anti-CD95 mAb, or recombinant TRAIL (Fig. 4A). Caspase-2 processing was detectable in response to all these stimuli, although the response to γ radiation was quite modest. The Bid mutant blocked Caspase-2 processing induced by etoposide and γ radiation, but left the death receptor response partially unaffected. In the presence of dn Caspase-9, Caspase-2 processing in response to etoposide and γ radiation was also abrogated, arguing that it was dependent on mitochondrial activation. Again, the response to death receptors was partially unaffected (Fig. 4B). However, both dn FADD and c-FlipL drastically inhibited the Caspase-2 processing in response to CD95 and TRAIL receptor stimulation (Fig. 4C). This indicates that Caspase-2 processing in the death receptor pathway is dependent on Caspase-8/10 activation. The collective data allow us to conclude that detectable Caspase-2 processing in response to etoposide and γ radiation or death receptor stimulation occurs downstream of inducer caspase activation by respectively, the mitochondria or the death receptor complex.
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**Figure 4** - Caspase-2 processing occurs downstream of Caspase-9 in the DNA damage pathway and independent of Caspase-9 in the death receptor pathway. A, J16 cells, transduced with empty vector or Bid D60E/D75E cDNA were stimulated with etoposide (E), γ radiation (IR), anti-CD95 mAb or recombinant TRAIL for the time periods outlined in Experimental Procedures or left untreated (M). Total lysates were separated on SDS-polyacrylamide gel and Caspase-2 processing was monitored by immunoblotting. Arrows mark the Caspase-2 proform and asterix its digestion products. B, J16 cells transduced with empty vector or dn Caspase-9 cDNA were treated for the indicated time periods and assayed as in A. C, J16 cells transduced with empty vector or dn FADD or c-FlipL cDNA were treated for the indicated time periods and assayed as in A.

**Figure 5** - Caspase-2 is not required for apoptosis-induction by etoposide and γ radiation. A, Caspase-2 knock down. Two different Caspase-2 siRNA constructs or empty vector were transduced into J16 cells stably expressing the ecotropic retroviral receptor. Total lysates were separated by SDS-PAGE and Caspase-2 protein levels were monitored by immunoblotting. Actin serves as a loading control. ECL values were quantified using a Fluorchem 8000 chemoluminescence imager and the average of the integrated density values (AVG) are indicated. B, J16 cells containing the Caspase-2 si II construct and control cells were treated with the indicated concentrations of etoposide and doses of γ radiation. Apoptosis was read out as nuclear fragmentation. Data are representative of two independent experiments and show ± S.D. from triplicate samples in one experiment.

Caspase-2 is not required for the apoptotic response to DNA damage, but does contribute to death receptor-induced apoptosis

The observation that Caspase-2 processing was only observed downstream of inducer caspases did not fully exclude the possibility that it might be involved in Bid processing upstream of the mitochondria in the DNA damage pathway. There is evidence that Caspase-2 can be activated in the absence of its proteolytic processing. To directly examine whether Caspase-2 was required for apoptosis-induction by DNA damaging regimens, we introduced a retroviral siRNA construct in J16 cells to stably
downregulate its expression. To enhance transduction efficiency, we used J16 cells expressing the ecotropic retroviral receptor. Two different siRNA constructs were tested, which had proven effective in transient transfection experiments in 293T cells. One of these constructs downregulated endogenous Caspase-2 to about 35% of its original level (Fig. 5A). All further experiments were done with this Caspase-2 si II construct. The reduction in Caspase-2 protein level had no impact at all on apoptosis outcome in response to DNA damage (Fig. 5B). However, we observed an about 50% reduction in apoptosis upon TRAIL stimulation when Caspase-2 protein levels were downregulated (Fig. 6A). This result demonstrated that the Caspase-2 knock down was functional and that Caspase-2 contributes to the apoptotic response to death receptor stimulation. However, it is apparently not essential for apoptosis-induction by the DNA damaging anti-cancer regimens in Jurkatt cells.

**Figure 6** - Caspase-2 contributes to death receptor-induced Caspase-3 processing and apoptosis, but not to Cyt c release. J16 cells, stably transduced to express the Caspase-2 si II knock down construct as described for Fig. 5 were treated with recombinant TRAIL. A, Apoptosis was read out by nuclear fragmentation after treatment with the indicated concentrations of TRAIL. Data represent means ± S.D. of duplicate samples from two independent experiments. B, In vivo Cyt c release was read out by immunoblotting of mitochondrion-free cytosols at the indicated time points after stimulation with TRAIL. C, The percentage of cells expressing active Caspase-3 was determined by immunofluorescence with specific antibody after 6 h treatment with the indicated concentrations of TRAIL. Data are representative of two independent experiments and show means ± S.D. from triplicate samples in one experiment.

To further map Caspase-2 in the death receptor pathway, we examined the effect of its downregulation on Cyt c release and Caspase-3 activation. Mitochondrion-free cytosols derived from TRAIL stimulated control and Caspase-2 knock down cells were analyzed for the presence of Cyt c. As shown in Fig. 6B, Caspase-2 downregulation did not affect Cyt c release at all. In contrast, generation of active
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Caspase-3 was consistently reduced in the Caspase-2 knock down cells treated with TRAIL (Fig. 6C). The collective data indicate that Caspase-2 does not contribute to effector caspase activation in the DNA damage pathway, but does so in the death receptor pathway, downstream of inducer caspase activity.

\[\text{Figure 7 - Granzyme B does not participate in apoptosis-induction by etoposide and } \gamma \text{ radiation. J16 cells were stably transduced with empty vector or vector encoding the serpins PI-9 (HA-tagged) or SPI-6 (VSV-tagged), which inhibit the serine protease Granzyme B. A, Total cell lysates were separated on SDS-polyacrylamide gel and subjected to immunoblotting to detect expression (anti-HA and anti-VSV) of the transduced serpins. B, J16 cells were treated with etoposide or } \gamma \text{ irradiated and incubated for the indicated periods of time. Apoptosis was read out as nuclear fragmentation. The experiment is representative of three. C, J16 cells were used as targets for the cytolytic T cell clones JS/J7 and JS/J29. Apoptosis was read out as nuclear fragmentation, as outlined in Experimental Procedures.}\]
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Granzyme B is not involved in Bid processing following DNA damage

Our experiments using c-Flip, dn Caspase-9, Capase-2 knock down and the pan-caspase inhibitor zVAD-fmk together make it unlikely that a caspase is involved in Bid processing following treatment of J16 cells with etoposide or γ radiation. One other known aspartate-specific protease is Granzyme B, a serine protease present in lysosomal compartments in cytolytic T lymphocytes. Although its intracellular localization is inconsistent with a role in cleavage of cytoplasmic proteins like Bid, it has recently been implicated in the response to DNA damaging anti-cancer regimens in myeloid malignancies. In these cells, Granzyme B protein levels increased in response to etoposide and γ radiation. In addition, Granzyme B was released from intracellular compartments into the cytosol. Given this potential scenario, we examined whether Granzyme B was responsible for Bid cleavage in response to treatment of J16 cells with etoposide and γ radiation. To this end, we transduced J16 cells with either SPI-6 or PI-9, which are the mouse and human homologues of a serine protease inhibitor (serpin) that can effectively inhibit Granzyme B. Retrovirus-mediated expression of these serpins was confirmed by Western blotting (Fig. 7A). However, it did not affect the apoptotic response to etoposide or γ radiation (Fig. 7B). As a positive control, we tested the effect of serpin expression on the sensitivity of J16 cells to apoptosis-induction by cytolytic T cell clones. Upon specific recognition of target cells by means of their T cell antigen receptor, cytolytic T cells exocytose lysosomal granules that contain Granzyme B and deliver this into the target cells with the aid of perforin (see 11). Fig. 7C shows that SPI-6 and PI-9 effectively inhibited J16 apoptosis as induced by two specific cytolytic T cells clones, JS/J7 and JS/J29. We conclude that Granzyme B is not involved in etoposide- and γ radiation-induced apoptosis in J16 cells and is therefore not responsible for Bid cleavage in this pathway. Therefore, Bid processing must be attributed to a presently undefined aspartate-specific protease.

DISCUSSION

The data collected in this study lead to the model depicted in Fig. 8. Two independent lines of evidence argue that Bid is the essential mediator of mitochondrial activation in the apoptotic response to etoposide and γ radiation in the T leukemic cells studied: depletion of Bid from cytosols of stimulated cells and use of a Bid mutant. In addition, the finding that tBid is the common intermediate in the death receptor and DNA damage pathways in Jurkat cells explains the cross-resistance to etoposide and γ radiation in Jurkat cells selected for CD95-resistance. Consistent with the present model, we have
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previously found that in the resistant cells a cytosolic factor prevents tBid from permeabilizing mitochondria.

This unambiguous functional implication of Bid in anti-cancer drug- and γ radiation-induced apoptosis is novel. Initial analysis of death pathways in Bid<sup>−/−</sup> mice only indicated a requirement for Bid in CD95-induced apoptosis in hepatocytes. Etoposide-induced apoptosis in Bid<sup>−/−</sup> mouse embryonic fibroblasts (MEF) appeared normal. However, it was reported recently that Bid<sup>−/−</sup> MEF have an impaired apoptotic response to adriamycin or 5-fluorouracil. In the same study, it was found that the Bid gene contains a p53 response element. In colon and spleen tissue sections derived from γ-irradiated mice, Bid mRNA was induced in a p53-dependent manner. These findings suggest a role for Bid as a sensor of DNA damage in at least some tissues. Interestingly, Bid<sup>−/−</sup> mice are prone to develop a malignancy resembling chronic myelomonocytic leukemia, identifying Bid as a tumor suppressor. Whether this reflects a role for Bid in DNA damage responses or in feedback control of cell proliferation remains to be established.

**Figuur 8** - Apoptosis signaling by DNA damaging anti-cancer regimens and death receptors in the p53 mutant T leukemic cells examined. In response to etoposide and γ radiation, pre-existing Bid protein conveys the apoptotic signal to the mitochondria, contingent upon its activation by an undefined protease, which is not Granzyme B or any known inducer caspase, but cleaves at aspartates 60 and/or 75. Inhibition of Bid by Bcl-2 abrogates the apoptotic response. Caspase-2 is activated downstream of Caspase-9 either directly or indirectly via effector caspases and does not detectably contribute to apoptotic execution. Upon death receptor activation, Caspase-8 is activated and cleaves Bid, which signals to the mitochondria. Inhibition of Bid by Bcl-2 strongly inhibits the apoptotic response, but does not fully abrogate it. Caspase-8 also processes Caspase-2, which contributes to Caspase-3 activation and apoptotic execution, but not to mitochondrial activation. Caspase-8 may also process Caspase-3 directly.
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The Jurkat cells used in our studies have one dysfunctional p53 allele as revealed in a yeast reporter assay (see Experimental Procedures). Functionally therefore, they are expected to display a p53-deficient phenotype, which is consistent with our observations. The apoptosis gene expression profile before and after etoposide treatment and γ radiation revealed only Puma as a DNA damage response gene in J16 cells among the array of apoptosis regulators tested. We could not reliably detect Puma protein by immunoblotting or metabolic labeling with antibodies, validated by us and others. Since studies in Puma−/− mice recently revealed that Puma is important for etoposide- and γ radiation-induced p53-dependent apoptosis in thymocytes, we further tested its involvement using a validated siRNA construct. However, we found no effect of Puma siRNA on etoposide- and γ radiation-induced apoptosis in J16 cells (results not shown).

Interestingly, tissue specific roles for Puma and Noxa are emerging, as Noxa was not required for thymocyte apoptosis in response to γ radiation, but made a significant contribution to intestinal crypt cell apoptosis. Puma was also found to contribute to p53-independent pathways, such as serum deprivation-induced apoptosis in myeloid cells. Clearly, division of labor between BH3-only proteins needs to be studied carefully for each apoptotic input in different tissues and their developmental stages. Systematic study of responses in Bid−/− mice will hopefully reveal in which primary cell lineage the pathway dominates that we have elucidated by this study. Since Jurkat T cells represent mature thymocytes, the pathway revealed here might be operational in cycling mature T cells, which die in response to etoposide and γ radiation in a p53-independent manner.

Proteolytic cleavage of Bid within the flexible loop formed between helix 2 and 3 reveals the hydrophobic residues in the BH3-domain helix 3 that trigger the apoptotic pathway. Substitution of aspartate by glutamate at residues 60 and/or 75 in this loop is not expected to alter its conformation. Therefore, we interpret the finding that the Bid mutant inhibits etoposide- and γ radiation-induced apoptosis to mean that aspartate-cleaved Bid is a signaling intermediate in these pathways. The alternative explanation, that mutated Bid represses the DNA damage pathway due to an acquired alternative conformation and resulting activity, is considered unlikely. Unfortunately, in Jurkat cells the amount of Bid that is proteolytically processed upstream of the mitochondria is below detection levels, even in the death receptor pathway. The Bid processing that can be detected is the result of a mitochondrial feedback loop, since it is fully abrogated by Bcl-2 overexpression. Therefore, we cannot validate the effect of the Bid mutant on endogenous Bid processing in vivo. However, its dramatic effect on both death receptor- and DNA damage-induced Cyt c release strongly suggests that it acts in a dominant negative manner.
Several groups have reported links between Caspase-2 and release of mitochondrial pro-apoptotic factors. The finding that Caspase-2 resides in the nucleus and can signal from this site to the mitochondria, make it an attractive candidate to control the DNA damage response. Indeed, Lassus et al. have presented an elegant study with siRNA that indicates an important contribution of Caspase-2 to etoposide-induced apoptosis in U2OS sarcoma cells, A549 carcinoma cells and E1A expressing IMR90 fibroblasts. Nevertheless, using a similar siRNA approach to downregulate Caspase-2, we could not demonstrate such a contribution in Jurkat T cells. Lymphoblasts from Caspase-2−/− mice have a normal apoptotic response to etoposide, and thymocytes have a normal response to γ radiation, suggesting that the contribution of Caspase-2 to the DNA damage pathway may be lineage-specific. At present, we cannot reconcile our findings with those of Robertson et al., who found by using tetrapeptide inhibitors and transfection with Caspase-2 anti-sense construct that Caspase-2 is involved in mitochondrial activation during etoposide-induced apoptosis in Jurkat cells.

The effect of siRNA for Caspase-2 on TRAIL-induced apoptosis served as a positive control in our study. Our findings agree with those of Droin et al., who downregulated Caspase-2 in Jurkat cells with an antisense construct. These authors also found a partial reduction in the apoptotic response to CD95 and TRAIL receptor stimulation. However, they concluded that Caspase-2 was involved in Caspase-8 activation and ensuing Bid cleavage, since these events were affected by Caspase-2 downregulation. However, as mentioned above, the Caspase-8 and Bid processing that is detectable in Jurkat cells, is the resultant of a mitochondrial feedback loop. Therefore, our interpretation that Caspase-2 acts downstream from Caspase-8, as based on c-FlipL overexpression, is most likely the correct one (Fig. 8).

Caspase-8, Caspase-2, and Caspase-3 are known to cleave Bid at Asp-60 (Asp-59 in murine Bid), while Granzyme B cleaves Bid at Asp-75. We have excluded Caspase-8, -2 (and -9), as well as Granzyme B from cleaving Bid in the DNA damage pathway. Therefore, the search is on for an aspartate-specific protease, which can act as inducer in this pathway. We have previously reported that the pan-caspase inhibitor zVAD could not block Cyt c release in response to etoposide and γ radiation in J16 cells. This finding argues against Caspase-3 or other caspases playing a role in this pathway. Moreover, effector caspases are unlikely candidates because they require aspartate-specific cleavage to become activated themselves. To our knowledge, no other aspartate-specific proteases than caspases and Granzyme B have been defined in mammalian cells. The cysteine protease calpain has been implicated in radiation-induced apoptosis in thymocytes, but calpain cleaves Bid at Gly-70. Lysosomal
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extracts cleave Bid at Arg-65$^{52}$. Since the mutant Bid protein used in this study seems to act as a dominant negative, it may bind with high affinity to the upstream protease involved in its cleavage, which should allow its identification.

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Supplemental Figure - Apoptosis gene expression profile by MLPA of etoposide treated and γ-irradiated cells. A, J16 cells were treated with etoposide for 0, 3, 6, or 9 h before total RNA was isolated. The apoptosis gene expression profile is shown in four subgroups: BH3-only proteins, Bax-type proteins, Bcl-2-type proteins and other apoptosis regulators. Expression of β2-microglobulin is shown here as representative of three housekeeping genes whose expression did not alter throughout the time courses. Bars represent peak areas of GeneScan profiles, normalized to percentage of total signal per sample. B, like A, but cells were γ-irradiated.
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