Cephalostatin 1 Selectively Triggers the Release of Smac/DIABLO and Subsequent Apoptosis That Is Characterized by an Increased Density of the Mitochondrial Matrix

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ABSTRACT

Cephalostatin 1 is a bis-steroidal marine natural product with a unique cytotoxicity profile in the in vitro screen system of the National Cancer Institute, suggesting that it may affect novel molecular target(s). Here we show that cephalostatin 1 induces a novel pathway of receptor-independent apoptosis that selectively uses Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with a low isoelectric point) as a mitochondrial signaling molecule. At nanomolar concentrations, cephalostatin 1 triggers dose- and time-dependent DNA fragmentation in leukemia Jurkat T cells. Apoptosis was found to be dependent on caspase activity because the pan-caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone blocks cephalostatin 1-mediated DNA fragmentation. The CD95 death receptor as well as other caspase-8–requiring death receptors were not involved because Jurkat T cells lacking the CD95 receptor or caspase-8 and control cells responded equally to cephalostatin 1. Although cephalostatin 1 affects mitochondria by dissipating the mitochondrial membrane potential, neither cytochrome c nor apoptosis-inducing factor is released, as shown by Western blot analysis. Interestingly, cephalostatin 1 selectively triggers the mitochondrial release of the inhibitor of apoptosis antagonist Smac/DIABLO. Overexpression of the antiapoptotic protein Bcl-xL delayed both Smac/DIABLO release and onset of apoptosis, suggesting that Smac/DIABLO is required for cephalostatin 1–induced apoptosis. This new mitochondrial pathway is accompanied by marked structural changes of mitochondria as shown by transmission electron microscopy.

INTRODUCTION

Cephalostatins are remarkable antineoplastic bis-steroidal natural products showing activity in the subnanomolar to nanomolar range. Cephalostatin 1, the first member of this series of compounds (cephalostatin 1–19), was detected in 1972 and subsequently isolated from extracts of Cephalodiscus gilchristi, a small Southeast African marine worm. The structure was reported in 1988 (1). In the 60-cell line in vitro screen of the National Cancer Institute, cephalostatin 1 yielded a mean panel 50% growth inhibition concentration of approximately 1 nM (2). Cephalostatin 1 is now in preclinical development. However, the understanding of the antineoplastic activity of cephalostatin 1 at the molecular level has remained elusive.

A wide range of chemotherapeutic drugs induce death in malignant cells by triggering apoptosis (3). Two major apoptotic pathways used by cytotoxic drugs have been described: one that begins with the ligation of death receptors and subsequently activates the initiator caspase-8; and another that integrates various cell stress signals via the “activation” of mitochondria. One crucial event in the mitochondrial pathway is the release of cytochrome c from the mitochondria into the cytosol. Once released, cytochrome c initiates the assembly of apoptotic protease-activating factor 1 (Apaf-1) and procaspase-9 into a holoenzyme complex called the “apoptosome,” which in turn activates the initiator caspase-9 (4). Initially activated caspase-8 (receptor-mediated pathway; extrinsic pathway) or caspase-9 (mitochondrial pathway; intrinsic pathway) activates downstream caspases, such as caspase-3, the activation of which results in DNA fragmentation and apoptosis.

A current view of drug-induced apoptosis emphasizes the role of different cell organelles [e.g., mitochondria, cytoskeleton, nucleus, plasma membrane, lysosomes, and the endoplasmic reticulum (ER)] as stress sensors that either reroute the signal directly through mitochondria or in some cases activate their own death pathways (5, 6). The central role of mitochondria in drug-induced apoptosis implies that alterations in this particular pathway play an important role in drug resistance. Indeed, resistance to chemotherapy was found to be related to increased levels of the mitochondria-protecting proteins Bcl-2 and Bcl-xL (4, 7, 8). Besides these, inhibitors of apoptosis proteins (IAPs) are shown to be overexpressed in cancer and are associated with resistance to apoptosis (7, 9). Some IAP family proteins bind and inhibit the activation of the initiator caspase-9 as well as activated effector caspases-3 and -7 (4, 9) and are thus endogenous repressors of the mitochondrial pathway of apoptosis. IAPs are antagonized by Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP-binding protein with a low isoelectric point). Mature Smac/DIABLO is a homodimer of two 21-kDa triple α-helical bundle monomers that reside in the mitochondrial intermembrane space. It is released during apoptosis into the cytosol, where it can interact with IAPs, thereby promoting caspase activation (10).

New drugs that induce apoptosis by mechanisms differing from those of classical chemotherapeutic drugs might provide a chance to overcome chemoresistance. The unique differential cytotoxicity profile of cephalostatins obtained from the 60-cell line in vitro screen of the National Cancer Institute provides a good possibility that cephalostatins may affect novel molecular targets (2). Therefore, the aims of the present study were (a) to examine whether cephalostatin 1 induces apoptosis in leukemia Jurkat T cells and (b) to focus on the characterization of the apoptotic pathways involved.

MATERIALS AND METHODS

Compounds. Cephalostatin 1 was isolated from C. gilchristi (order, Cephalodiscida) as described previously (1). Purity of the compound was at least 98% as judged by high-performance liquid chromatography analysis. Before application, cephalostatin 1 was dissolved and further diluted in DMSO. Final DMSO concentration did not exceed 1%, a concentration verified not to interfere with the experiments performed. Etoposide, staurosporine, and the caspase inhibitors were purchased from Calbiochem (Bad Soden, Germany). The soluble CD95 ligand was from Alexis Biochemicals (Grün-
berg, Germany), and propidium iodide (PI) was obtained from Sigma (Deisenhofen, Germany).

**Cell Culture.** Human leukemia Jurkat T cells (clone J16), the CD95-resistant JurkatR (11), Jurkat T cells transfected with vector control or Bcl-xL (12), Jurkat T cells (clone A3), and caspase-8-deficient A3 (Ref. 13; kindly provided by Drs. P. H. Krammer and H. Waleczak (Heidelberg, Germany) and Dr. K. Schulze-Osthoff (Münster, Germany)] were cultured (37°C and 5% CO₂) in RPMI 1640 containing 2 mM L-glutamine (PAN Biotech, Aidenbach, Germany) supplemented with 10% FCS (PAA Laboratories, Colbe, Germany). Medium of transfected cells was supplemented with 1 mg/ml G418 (Life Technologies, Inc., Eggenstein, Germany) every fifth passage.

**Cell Viability.** Impaired cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, based on the ability of viable cells to reduce yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to blue formazan, as described previously (14).

**Detection and Quantification of Apoptosis.** Apoptosis was judged by (a) the visualization of apoptotic nuclei after staining with Hoechst 33342 (Sigma) by fluorescence microscopy and (b) translocation of phosphatidylserine to the cell surface using an annexin V-FITC apoptosis detection kit (Calbiochem). Quantification of apoptosis was performed as described by Nicolletti et al. (15). Briefly, cells were incubated for 24 h in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml PI) and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Nuclei to the left of the G1 peak containing hypoploid DNA were considered apoptotic.

**Analysis of Mitochondrial Transmembrane Potential (ΔΨm).** Cells were stained with the fluorochrome JC-5, 6,6-3,3-dethacrolone-1,1,3,3-tetramethylbenzimidazol carbonyl cyanide [JC-1 (1.25 μM/ml); Molecular Probes, Eugene, OR] as described by Bernardi et al. (16) and Cossarizza et al. (17). The membrane potential was measured by fluorescence-activated cell-sorting (FACS) analysis. JC-1 aggregates were detected at 585 nm (FL-2), and JC-1 monomers were detected at 530 nm (FL-1). The percentage of cells with true green fluorescence was calculated by gate analysis on a FACSCalibur using CellQuest software (Becton Dickinson).

**Measurement of Cytochrome c Release.** Release of cytochrome c from mitochondria was analyzed as described by Leist et al. (18). Briefly, cell pellets were resuspended in permeabilization buffer [210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM succinyl, 0.2 mM EGTA, 0.15% BSA, and 60 μg/ml digitonin (pH 7.2, 4°C)] and incubated for 20 min at 4°C. Permeabilized cells were centrifuged (300 × g), and the supernatant was removed and centrifuged again (10 min; 13,000 × g). The obtained cytosol was separated by 15% SDS-PAGE and probed for cytochrome c as described below. The remaining pellet of permeabilized cells was lysed in 0.1% Triton/ PBS (15 min; 4°C) and centrifuged (12,000 × g; 4°C; 10 min), and the supernatant containing mitochondrial cytochrome c was analyzed by SDS-PAGE.

**Western Blot Analysis and Immunoprecipitation.** Cells were collected by centrifugation, washed with ice-cold PBS, and lysed in 1% Triton X-100, 0.15 M NaCl, and 30 mM Tris-Cl (pH 7.5) with the protease inhibitor Complete (Roche, Mannheim, Germany) for 30 min. Lysates were homogenized with a 22-gauge needle and centrifuged at 10,000 × g for 10 min at 4°C. Equal amounts of protein were separated by SDS-PAGE (7.5% SDS-PAGE for Apaf-1 and poly (ADP-ribose) polymerase, 10% SDS-PAGE for caspase-8 and caspase-9, 12% SDS-PAGE for caspase-3 and X-linked IAP (XIAP), 15% SDS-PAGE for cytochrome c) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Eschborn, Germany). Equal protein loading was controlled by Coomassie Blue staining of gels. Membranes were blocked with 5% fat-free milk powder in PBS containing 0.05% Tween 20 (1 h) and incubated with specific antibodies against caspase-3 (mouse IgG2a: BD Biosciences, Heidelberg, Germany), caspase-8 (mouse monoclonal antibody, clone SF7; Upstate Biotechnology, Lake Placid, NY), caspase-9 (mouse monoclonal IgG1, clone Ab-2; Oncogene, San Diego, CA), Apaf-1 (mouse IgG2a; BD Biosciences), XIAP (rabbit polyclonal antibody; Chemicon, Temecula, CA), poly (ADP-ribose) polymerase (mouse IgG1; Oncogene), cytochrome c (mouse monoclonal antibody 7H8.2C12; BD Biosciences), AIF (rabbit polyclonal antibody; Upstate Biotechnology), and Smac/DIABLO (Serotec, Oxford, United Kingdom) overnight at 4°C. Specific proteins were visualized by secondary antibodies conjugated to horseradish peroxidase and the Renaissance Plus reagent (NEN Life Science, Zaventem, Belgium). Pictures were taken on a Kodak Digital Science Image station 440CF (NEN Life Science).

**RESULTS**

**Cephalostatin 1 Induces Apoptosis in Jurkat Leukemia T Cells.** Initial dose-response studies using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay revealed that 1 μM cephalostatin 1 effectively impairs cell viability of Jurkat T cells (IC₅₀ = 1.25 nm). This cytotoxicity was attributable to the induction of apoptosis as shown by the following morphological and biochemical features: 1 μM cephalostatin 1 led to the characteristic apoptotic morphology with shrunken cells and apoptotic bodies (data not shown). Nuclei fragmentation was detected by fluorescence microscopy after DNA staining with Hoechst 33342 (Fig. 1A, left panel) and by the appearance of nuclei with subdiploid DNA content as shown by flow cytometry after PI staining (Fig. 1A, middle panel). Annexin V-FITC labeling revealed the exposure of phosphatidylserine on the outside of the plasma membrane (Fig. 1A, right panel).
Apoptosis induction by cephalostatin 1 occurred in a dose- and time-dependent manner. The appearance of apoptotic cells was significant 6 h after exposure to cephalostatin 1 (1 μM) and at a concentration as low as 1 nM (24 h; Fig. 1B).

Cephalostatin 1-Induced Apoptosis Is Dependent on the Activation of Caspases. To elucidate whether caspase activation is required for cephalostatin 1-induced apoptosis, cells were preincubated with the broad-spectrum caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk). Caspase inhibition led to a complete inhibition of cephalostatin 1-induced DNA fragmentation (Fig. 2A), proving the importance of caspases in this apoptotic process. To get a better idea of whether cephalostatin 1-triggered apoptosis follows the extrinsic pathway including activation of the initiator caspase-8 or the intrinsic pathway under involvement of mitochondria and the initiator caspase-9, we examined the activation of these caspases by Western blot analysis. Fig. 2B illustrates the time-dependent activation of caspase-8 by cephalostatin 1 (1 μM). The caspase-8 cleavage product p42/p44 did not appear until 16 h after stimulation. In contrast, the processing of the proform of caspase-9 to the active forms (p35/p37) occurred after only 4 h (Fig. 2C). To test whether caspase-3, an important effector caspase, is activated downstream of caspase-9, the processing of caspase-3 (Fig. 2D) and poly(ADP-ribose) polymerase cleavage (Fig. 2E) in response to cephalostatin 1 (1 μM) was demonstrated.

The CD95 Receptor and Caspase-8 Are Not Required for Apoptosis Induction by Cephalostatin 1. The extrinsic pathway involves death receptors such as the CD95 receptor, the activation of which processes caspase-8. To study whether the CD95 receptor is participating in cephalostatin 1-induced apoptosis, we treated control cells and CD95 receptor-deficient JurkatR cells (11) with cephalostatin 1 (0.1–1 μM; 24 h). In Jurkat cells, no reduction of apoptosis could be detected compared with control cells (Fig. 3A). Thus, the CD95 receptor is not required for cephalostatin-induced apoptosis. Because other death receptors also use caspase-8 as an initiator caspase, we examined whether caspase-8 activation is necessary for apoptosis or whether caspase-8 is merely activated downstream as a consequence of a caspase amplification loop in response to cephalostatin 1. Fig. 3B shows that control cells and cells deficient in caspase-8 (caspase-8−/− cells) respond equally to cephalostatin 1

![Fig. 1. Cephalostatin 1 induces apoptosis in Jurkat leukemia T cells. A, left panel, cells were treated with cephalostatin 1 (1 μM, 16 h) and subsequently stained with Hoechst 33342. Representative pictures of untreated (Control) and cephalostatin 1-treated cells are shown. Middle panel, FACS analysis of PI-stained nuclei of cells treated as described above for 24 h. Histograms show the distribution of nuclei according to their DNA content. Counts left of the G1 peak (gated) demonstrate the appearance of nuclei with subdiploid DNA content. Right panel, FACS analysis of annexin V-FITC and PI-stained cells, either left untreated (Control) or stimulated with cephalostatin 1 (1 μM, 8 h). Cells appearing in the bottom right quadrant show positive annexin V-FITC staining that indicates phosphatidylserine exposure on the cell surface and no DNA staining with PI, proving intact cell membranes. B, cephalostatin 1-induced apoptosis is time and dose dependent. Cells were stimulated with increasing concentrations of cephalostatin 1 for 24 h (top panel) or cephalostatin 1 (1 μM) for the indicated periods of time (bottom panel). % Apoptotic cells, percentage of cells with subdiploid DNA content as described in "Materials and Methods." Bars, mean ± SE of three independent experiments performed in triplicate; **, P < 0.001 (ANOVA/Dunnett’s test).](image)

![Fig. 2. Cephalostatin 1-induced apoptosis is dependent on the activation of caspases. A, inhibition of cephalostatin 1-induced apoptosis by the caspase inhibitor zVAD-fmk. Cells were left untreated (Control), incubated with cephalostatin 1 (1 μM, 24 h), or pretreated with zVAD-fmk (25 μM, 1 h) and then stimulated with cephalostatin 1 (1 μM, 24 h). Etoposide (E; 25 μg/ml, 24 h) was used as positive control. Apoptotic cells were quantified by flow cytometry as described in "Materials and Methods." B–E, representative Western blots showing time-dependent (0–24 h) cleavage as follows: B, procaspase-8 isoforms are cleaved to the p42 and p44 cleavage products; C, procaspase-9 is cleaved to the active cleaved forms p35 and p37; D, procaspase-3 is cleaved to p17; and E, 116-kDa PARP is cleaved to its p89 cleavage product after treatment with cephalostatin 1 (1 μM). Cells were lysed, and proteins were separated by SDS-PAGE as described in "Materials and Methods." All experiments were performed three times with consistent results. Bars, mean ± SE of three independent experiments performed in triplicate; **, P < 0.001 (ANOVA/Dunnett’s test). PARP, poly(ADP-ribose) polymerase.](image)

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(1 μM), suggesting that activation of caspase-8 is not required for cephalostatin 1-induced apoptosis. To verify that the used cell line does not express caspase-8, we performed Western blot analysis (data not shown) and treated Jurkat A3 (control cells) and Jurkat caspase-8−/− cells with soluble CD95 ligand or etoposide (25 μg/ml). As expected, Jurkat caspase-8−/− cells were protected against CD95 ligand but not against etoposide (Fig. 3C). These results, together with the fact that caspase-8 succeeds caspase-9 activation, clearly show that cephalostatin 1 does not induce apoptosis via the extrinsic pathway.

**Cephalostatin 1 Induces Inner Mitochondrial Membrane Permeabilization and Release of Smac/DIABLO but not Release of Cytochrome c or AIF.** The observed early activation of the initiator caspase-9 in response to cephalostatin 1 (Fig. 2) points to a putative mitochondria-mediated signaling. Hallmarks of the mitochondrial pathway of apoptosis (intrinsic pathway) are the release of cytochrome c from the mitochondrial intermembrane space into the cytosol and the dissipation of the electrochemical gradient (ΔΨm) on the inner mitochondrial membrane (inner mitochondrial membrane permeabilization). Fig. 4A shows that cephalostatin 1 (1 μM) induces inner mitochondrial membrane permeabilization in Jurkat T cells as measured by flow cytometry using the fluorochrome JC-1. The first
significant dissipation of $\Delta \psi_{m}$ was evident 8 h after stimulation. Most surprisingly, cephalostatin 1 (1 $\mu$m; 0–24 h) does not trigger the release of cytochrome c into the cytosol. Etoposide (25 $\mu$g/ml) was used as positive control (Fig. 4B). Thus, cephalostatin 1 activates caspase-9 independently of cytochrome c.

Besides cytochrome c, additional proapoptotic factors present within mitochondria and released after various apoptotic stimuli are the AIF and Smac/DIABLO (4). Fig. 4C demonstrates that similar to cytochrome c, AIF is not released in response to cephalostatin 1. In striking contrast, cephalostatin 1 triggers an early (2 h) and intense release of Smac/DIABLO that is not abrogated by the pan-caspase inhibitor zVAD-Fmk. This indicates that cephalostatin 1-induced Smac/DIABLO release is an early event that occurs before and independent of caspase activation.

This unique property (i.e., the selective release of Smac/DIABLO) of cephalostatin 1 was confirmed by immunofluorescence experiments detecting cytochrome c, Smac/DIABLO, and AIF in response to cephalostatin (1 $\mu$m) and etoposide (25 $\mu$g/ml) by confocal laser scanning microscopy. Fig. 4E demonstrates that in untreated Jurkat cells, cytochrome c and Smac/DIABLO colocalize at distinct areas (mitochondria). In response to cephalostatin, Smac/DIABLO is released, whereas cytochrome c still displays a punctuate mitochondrial appearance. In contrast, etoposide leads to a diffuse pattern of both cytochrome c and Smac/DIABLO. Similar to Western blot analysis (Fig. 4C), AIF translocation was not detected and was hardly detected in response to cephalostatin and etoposide, respectively.

**Overexpression of Bcl-xL Delays Smac/DIABLO Release and Onset of Cephalostatin-Induced Apoptosis.** To clarify whether Smac/DIABLO is required for cephalostatin 1-triggered apoptosis, we examined whether overexpression of Bcl-xL, a mitochondria-protecting protein that is shown to prevent the release of Smac/DIABLO (19), also inhibits apoptosis. Initially, Jurkat/neO and Jurkat/bcl-xL cells were verified to differ in Bcl-xL expression by Western blot analysis (data not shown) and by their response to staurosporine (500 nM, 24 h [20, 21]). As seen in Fig. 5A, staurosporine readily kills Jurkat/neO cells, whereas Jurkat/bcl-xL cells are protected against staurosporine. Both cell types were then exposed to cephalostatin 1 (1 $\mu$m) for 0–24 h. Fig. 5B shows that Smac/DIABLO release is significantly delayed in Jurkat/bcl-xL cells compared with Jurkat/neO cells (2 versus 16 h). Corresponding to this delayed Smac/DIABLO release, onset of DNA fragmentation in response to cephalostatin 1 is delayed (8 h for Jurkat/neO versus 24 h for Jurkat/bcl-xL; Fig. 5C). Interestingly, dissipation of $\Delta \psi_{m}$ was not affected by overexpression of Bcl-xL (Fig. 5D). This observation, together with its late appearance (16 h), indicates that dissipation of $\Delta \psi_{m}$ is a secondary event within the cephalostatin 1-induced signaling cascade.

**Caspase-9 Is Not Recruited into an Apaf-1-Containing Apoptosome and Does Not Act as a Major Initiator Caspase Activated in Response to Cephalostatin 1.** Next, we sought to clarify whether, in the absence of cytochrome c, cephalostatin 1-activated caspase-9 still acts as essential initiator caspase. Therefore, we examined whether caspase-9 is recruited into the apoptosome by binding to Apaf-1 in cephalostatin 1-activated cells. Western blot analysis of caspase-9 immunoprecipitates (Fig. 6A) shows that in etoposide (25 $\mu$g/ml)-activated cells, caspase-9 associates with Apaf-1, whereas cephalostatin (1 $\mu$m) treatment does not lead to caspase-9-Apaf-1 association. This suggests that cephalostatin 1 treatment does not induce the formation of an apoptosome. This outcome posed the question of whether caspase-9 is still essential for cephalostatin 1-mediated apoptosis. Inhibition of caspase-9 by the caspase-9 inhibitor benzoyloxycarbonyl-Leu-Glu(OMe)-His-Asp(OMe)-fluoromethylketone (50 $\mu$m) led to an inhibition of etoposide-induced caspase-3 activity (Fig. 6B) but not to a complete inhibition of cephalostatin 1-induced apoptosis (Fig. 6C). This result indicates that cephalostatin 1-activated caspase-9 contributes to but is not essential for cephalostatin 1-mediated apoptosis.

**Cephalostatin 1-Released Smac/DIABLO Displaces XIAP from Caspase-9.** Smac/DIABLO is suggested to act as an apoptosis promoter by interacting with IAPs, preventing these proteins from bind-
Methods.

Bars of proapoptotic mitochondrial molecules such as cytochrome c, released in response to cephalostatin 1 is able to dissociate XIAP from caspase-9 (Fig. 7). During stress-induced apoptosis, Smac/DIABLO is released along with cytochrome c from mitochondria into the cytosol. Although released cytochrome c contributes to the formation of the apoptosome and thereby to the initiation of the caspase-9-dependent caspase cascade, Smac/DIABLO promotes caspase activity by binding to the XIAP, and probably other IAPs, in a manner that displaces caspases from their inhibitor XIAP (24, 25). The selective release of Smac/DIABLO as a main mitochondrial signaling molecule. The following observations support this view: (a) cephalostatin 1-induced apoptosis occurred independently of the CD95 death receptor and caspase-8; (b) cephalostatin 1-triggered DNA fragmentation was significantly reduced after overexpression of the Bcl-xL protein; (c) despite this fact, cephalostatin 1 did not promote the release of cytochrome c or AIF (however, it led to an early and intense release of functionally active Smac/DIABLO that occurred before and independent of caspase activation); and (d) overexpression of Bcl-xL delayed both Smac/DIABLO release and onset of apoptosis. Although caspase-9 was activated and released from XIAP in response to cephalostatin, no apoptosome formation could be detected. Moreover, caspase-9 did not seem to be the essential initiator caspase in cephalostatin-mediated apoptosis. This unique mitochondria-involving signaling pathway was morphologically characterized by the appearance of mitochondria with an increased matrix density.

Chemotherapeutic drug-induced cellular stress or damage is sensed by different cellular organelles, such as mitochondria, the cytoskeleton, the nucleus, the ER, or the plasma membrane. Most signals are finally integrated by mitochondria that translate these diverse signals into a common execution pathway by the release of cytochrome c, the formation of the apoptosome, and, finally, the activation of caspases. The cellular signal generated by cephalostatin 1 seems to use mitochondria but does not culminate in the common execution pathway triggered by cytochrome c. The cues involved in cephalostatin 1-triggered signaling upstream of mitochondria do not involve the CD95 death receptor or caspase-8, as shown by Jurkat T cells deficient in these molecules. The requirement of other active caspases upstream of mitochondria is also unlikely because mitochondrial release of Smac/DIABLO was not affected by the pan-caspase inhibitor zVAD-fmk. Although the signaling leading to mitochondrial Smac/DIABLO release in response to cephalostatin 1 has not yet been identified, it does not include caspase activation. This observation is in contrast to a recently proposed model in which the release of Smac/DIABLO from mitochondria is a secondary event following cytochrome c release and caspase activation (23). These differing results, however, may be attributable to the different apoptotic stimuli used.

During stress-induced apoptosis, Smac/DIABLO is released along with cytochrome c from mitochondria into the cytosol. Although released cytochrome c contributes to the formation of the apoptosome and thereby to the initiation of the caspase-9-dependent caspase cascade, Smac/DIABLO promotes caspase activity by binding to the XIAP, and probably other IAPs, in a manner that displaces caspases from their inhibitor XIAP (24, 25). The selective release of Smac/DIABLO is triggered by a death receptor-independent apoptotic pathway in the Jurkat T cells that uses mitochondria as signaling integrator and inhibiting caspases (10). To clarify whether Smac/DIABLO released in response to cephalostatin 1 is able to dissociate XIAP from caspase-9, we immunoprecipitated Smac/DIABLO (Fig. 7A) and caspase-9 (Fig. 7B), respectively, and detected bound XIAP by Western blot analysis. Fig. 7, A and B, demonstrates that in untreated cells (CO), XIAP is associated with caspase-9 but not with Smac/DIABLO. Cephalostatin (1 μM) as well as etoposide (25 μg/ml) treatment led to an association of XIAP with Smac/DIABLO and a dissociation of XIAP from caspase-9.

Cephalostatin 1 Leads to Structural Changes in Mitochondria. Mitochondrial swelling and subsequent rupture of the outer mitochondrial membrane are considered one mechanism mediating the release of proapoptotic mitochondrial molecules such as cytochrome c (22). Because cephalostatin 1 induces the release of Smac/DIABLO, but not that of cytochrome c and AIF, we examined the ultrastructural changes of mitochondria in Jurkat cells after stimulation with 1 μM cephalostatin 1 (16 h). As depicted in Fig. 8, cells treated with cephalostatin 1 show mitochondria that appear smaller with increased electron density compared with untreated cells, suggesting a “condensation” of the mitochondrial matrix. The cristae appear blurred. In close vicinity to the mitochondria, a great number of vesicles, presumably ER-derived, were observed.

DISCUSSION

This is the first report showing that an experimental chemotherapeutic drug triggers a death receptor-independent apoptotic pathway in leukemia Jurkat T cells that uses mitochondria as signaling integrator and

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Fig. 6. A, cephalostatin 1 treatment does not lead to association of caspase-9 with Apaf-1. Cells were either left untreated (CO) or stimulated with cephalostatin 1 (CPH; 1 μM) or etoposide (ETO; 25 μg/ml) for the indicated time, and lysates were immunoprecipitated (IP) with anti-caspase-9 antibody, followed by immunoblotting (IB) with anti-Apaf-1 (top panel) or anti-caspase-9 antibody (bottom panel). Results are representative of three Western blots. B and C, inhibition of caspase-9 does not lead to complete inhibition of cephalostatin 1-induced apoptosis. Cells were either left untreated (CO) or activated with cephalostatin (CPH; 1 μM) or etoposide (ETO; 25 μg/ml) in the absence or presence of the caspase-9 inhibitor zLEHD-fmk (50 μM, 1 h pretreatment) for 16 h. Then caspase-3 activity (B) or apoptosis (C) was quantified as indicated in “Materials and Methods.” Bars, mean ± SE of three independent experiments performed in triplicate. *** P < 0.001 (ANOVA/Dunn’s test).

Fig. 7. In response to the cephalostatin 1 released, Smac/DIABLO sequesters XIAP bound previously to caspase-9. Cells were either left untreated (CO) or stimulated with cephalostatin 1 (CPH; 1 μM) or etoposide (ETO; 25 μg/ml) for 4 h. A, lysates were immunoprecipitated (IP) with anti-Smac antibody, followed by immunoblotting (IB) with anti-XIAP (top panel) or anti-Smac antibody (bottom panel). B, lysates were immunoprecipitated (IP) with anti-caspase-9 antibody and analyzed by IB with anti-XIAP antibody (top panel) or anti-caspase-9 antibody (bottom panel). All experiments were performed three times with analogous results. Representative Western blots are shown.
DIABLO by cephalostatin 1 without cytochrome c and subsequent apoptosome formation are unusual events and raise the following question: how is caspase-9 activated in the absence of an apoptosome? Recent data suggest that under certain conditions, Smac/DIABLO may be sufficient to initiate a caspase-9-dependent caspase cascade in the absence of cytochrome c and apoptosome formation (26, 27). Our studies, however, promote the idea that in the absence of an apoptosome, the common mitochondrial execution pathway mediated by the initiator caspase-9 may not be essential for triggering apoptosis. A Smac-dependent and apoptosome-independent pathway was recently proposed for receptor-mediated apoptosis in “type II” cells, including Jurkat T cells. In type II cells, death receptor-mediated activation of caspase-8 was shown to lead to an incomplete processing of caspase-3. This inactive form is bound and thus prevented from autocatalytic processing by XIAP. Smac/DIABLO release was necessary and sufficient to promote full caspase-3 activation (19, 28). In this setting, Smac/DIABLO clearly functions as a promoter of caspase activation and not as an inducer. Full caspase-3 activation required both signaling via caspase-8 and Smac/DIABLO. This gives rise to the speculation that cephalostatin 1-induced apoptosis that is obviously routed via mitochondria may use other pathways that act in concert with Smac/DIABLO to fully activate a caspase cascade that finally leads to apoptosis. It is conceivable that other organelles, such as the ER, are involved in cellular stress sensing (29, 30). In our model, overexpression of Bcl-xL blocked both Smac/DIABLO release and apoptosis, indicating that Smac/DIABLO is a necessary molecule in cephalostatin 1-induced apoptosis; however, other molecules (located in mitochondria as well as in other organelles) with activities that are blocked by Bcl-xL may contribute to the activation of caspases and subsequent apoptosis.

The unique mode of mitochondrial “activation,” i.e., the selective release of Smac/DIABLO in response to cephalostatin 1, prompted us to examine putative changes in the mitochondrial morphology. Surprisingly, we found shrunken mitochondria with a higher matrix density after cephalostatin 1 treatment. This is in contrast to earlier observations made during “classical” mitochondrial apoptosis (e.g., such as that induced by staurosporine in Jurkat T cells). Here, apoptosis was shown to be accompanied by mitochondrial swelling (31). The observation of mitochondrial swelling during apoptosis is included in one hypothesis explaining how Bcl-2 family members mediate the release of proapoptotic mitochondrial proteins, such as cytochrome c: proapoptotic Bcl-2 family members alter mitochondrial homeostasis, resulting in organelle swelling, physical rupture of the outer membrane, and subsequent release of intermembrane proteins into the cytosol (22). This hypothesis obviously does not hold true for the release of Smac/DIABLO in response to cephalostatin 1 in Jurkat
T cells. However, the obvious difference in morphology of mitochondria after treatment with cephalostatin 1 emphasizes a role for mitochondria and that this role differs from that in classical mitochondrial apoptosis induced by chemotherapeutic drugs and compounds such as staurosporine.

In summary, we have presented evidence that the marine natural product cephalostatin 1 triggers apoptosis in leukemia Jurkat T cells by a novel pathway that promotes the release of Smac/DIABLO but not cytochrome c or AIF from mitochondria. Although, at present, it cannot be excluded that one or more other factors are involved that cooperate with Smac/DIABLO, Smac/DIABLO seems to be required for cephalostatin 1-induced apoptosis. Of course, strategies to silence Smac/DIABLO have to be established to finally answer this question. This unique mechanism is accompanied morphologically by mitochondrial condensation instead of swelling.

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