Essential role of mitochondria in apoptosis of cancer cells induced by the marine alkaloid Lamellarin D

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Abstract

Lamellarin D, a potent cytotoxic marine alkaloid, exerts its antitumor action through two complementary pathways: a nuclear route via topoisomerase I inhibition and a mitochondrial targeting. The present study was designed to investigate the contribution of these two pathways for apoptosis in cancer cells. Lamellarin D promoted nuclear apoptosis in leukemia cells without prominent cell cycle arrest. Signals transmitted by lamellarin D initiated apoptosis via the intrinsic apoptotic pathway. The drug induced conformational activation of Bax and decreased the expression levels of antiapoptotic proteins Bcl-2 and cIAP2 in association with activation of caspase-9 and caspase-3. Upon lamellarin D exposure, Fas and Fas-L expression was not modified in leukemia cells. Moreover, leukemia cells deficient in caspase-8 or Fas-associated protein with death domain underwent apoptosis through the typical mitochondrial apoptotic cascade, indicating that cell death induced by lamellarin D was independent of the extrinsic apoptotic pathway. Lamellarin D also exerted a topoisomerase I-mediated DNA damage response resulting in H2AX phosphorylation, and the upregulation of the DNA repair protein Rad51 and of p53, as well as the phosphorylation of p53 at serine 15. However, lamellarin D killed efficiently mutated p53 or p53 null cancer cells, and sensitivity to lamellarin D was abrogated neither by cycloheximide nor in enucleated cells. Lamellarin D–induced cytochrome c release occurs independently of nuclear factors in a cell-free system. These results suggest that lamellarin D exerts its cytotoxic effects primarily by inducing mitochondrial apoptosis independently of nuclear signaling. Thus, lamellarin D constitutes a new proapoptotic agent that may bypass certain forms of apoptosis resistance that occur in tumor cells. [Mol Cancer Ther 2009;8(12): 3307–17]

Introduction

Lamellarins represent a family of hexacyclic pyrrole alkaloids originally derived from marine invertebrates and have been shown to exert antineoplastic effects (1). Among lamellarins, the natural compound lamellarin D (Fig. 1A) has been shown to inhibit a large panel of tumor cell types in vitro. At doses in the micromolar range, lamellarin D exhibits high apoptotic activities in leukemia cell lines (2, 3) characterized by the dissipation of the mitochondrial membrane potential (Δψm), the release of cytochrome c from mitochondria, and caspase-3 activation (2). The proapoptotic effects of lamellarin D can result from several mechanisms. Lamellarin D was first described to inhibit nuclear topoisomerase I (4). In vitro, lamellarin D induced DNA cleavage with an efficiency just slightly lower than the prototype topoisomerase I inhibitor camptothecin (5). Structure-activity studies indicate a good correlation between topoisomerase I inhibition and cytotoxicity of lamellarin D derivatives, leading to the suggestion that one of the proapoptotic targets of lamellarin D is in the cell nucleus (5). Nevertheless, its role in apoptosis still needs to be shown. Interestingly, topoisomerase I-mutated cancer cells are not totally resistant toward lamellarin D–induced apoptosis, suggesting that induction of apoptosis could depend on other intracellular targets. We have previously reported strong evidence that lamellarin D and its amino synthetic derivative PM031379 can trigger apoptosis through a direct increase in mitochondrial permeability (2). These results were obtained using a cell-free system of apoptosis in which supernatants of purified mitochondria treated with lamellarin D were mixed with isolated nuclei. However, we could not conclude that apoptosis in intact cells results exclusively from this direct action on mitochondria. Thus, how lamellarin D triggers cell death still deserves further investigation.

Most of the conventional anticancer treatments are thought to induce cell death through indirect activation of the mitochondria-dependent pathway of apoptosis, a pathway often found altered in drug-resistant cancer cells (6).
In most cases, chemotherapeutic drugs first interact with an intracellular target resulting in stress signals that secondarily converge to mitochondria and finally result in apoptotic cell death. The best-known intracellular target of conventional anticancer drugs is nuclear DNA. Numerous cytotoxic agents, including topoisomerase I and II inhibitors, primarily damage nuclear DNA. As a consequence, DNA insults, in the absence of efficient repair, lead to cell apoptosis through activation of complex molecular cascades (7). The tumor suppressor protein p53 is at the center of the process coupling DNA damage to the initiation of apoptosis. In response to DNA insults, intracellular levels of p53 increase and promote the transcription of several proapoptotic genes of the bcl-2 family such as the BH3-only genes, noxa, puma, and bax (7). Additionally, the translocation of p53 from the nucleus to the mitochondria is associated with cell death in a transcription-independent manner (8).

Following stress signals generated by conventional treatments, the permeability of mitochondrial membranes is increased, leading to the release of proapoptotic proteins which in turn initiate the caspase cascade and finally result in cell death (7). The ratio of proapoptotic (e.g., Bax, Bak, Noxa, Puma) and antiapoptotic members (e.g., Bcl-2, Bcl-xl, Mcl-1) of the Bcl-2 family affects the permeability of mitochondrial membranes, thereby determining the sensitivity of cells to apoptotic signals (9). Thus, the expression level of antiapoptotic Bcl-2 proteins correlates with the resistance to a large spectrum of chemotherapeutic agents (9).

In this study, we examined the molecular circuits through which lamellarin D promotes cancer cell apoptosis. In particular, we investigated the role of the cell nucleus in lamellarin D–induced apoptosis. An understanding of the mechanisms by which lamellarin D causes apoptosis would permit identification of the most valuable molecularly targeted strategy for drug development.

**Materials and Methods**

**Chemicals**

Camptothecin was purchased from Sigma, and lamellarin D was synthesized at Pharmamar. Several caspase inhibitors were used: the pan–caspase inhibitor benzoylcarbonyl-Val-Ala-Asp(OME)-fluoromethylketone (z-VAD.fmk; Sigma), the caspase-1 inhibitor benzoylcarbonyl-Tyr-Val-Ala-Asp-chloromethylketone (z-YVAD.fmk; Bachem), the caspase-2 inhibitor benzoylcarbonyl-Val-Ala-Asp-fluoromethylketone (z-VDVAD.fmk; Calbiochem, Chemicon International), the caspase-3 inhibitor benzoylcarbonyl-Val-Glu-Val-Ala-Asp-chloromethylketone (z-DEVD.fmk; Pharmingen BD Biosciences), the caspase-8 inhibitor benzoylcarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (z-IETD.fmk; 50 μmol/L; Pharmingen BD Biosciences), and the caspase-9 inhibitor benzoylcarbonyl-Leu-Glu-His-Asp-fluoromethylketone (z-LEHD.fmk; Pharmingen BD Biosciences). 4′,6-Diamidino-2-phenylindole (DAPI) and tetramethylrhodamine methyl ester (TMRM) were purchased from Molecular Probes. Other compounds were from Sigma.

**Cell Lines**

The mouse leukemia P388 cell line and P388CPT5, its topoisomerase I–mutated subclone (kindly provided by Pr. J.-F. Riou, Museum National d’Histoire Naturelle, Paris, France), were cultured as described (2). 2B411 T hybridoma cells stably transfected with a SFFVneo vector containing the human bcl-2 gene or the neomycin resistance gene (neo) only (kindly provided by Dr. Jonathan Ashwell, NIH) and the cell lines Jurkat, Jurkat I 9.2 (caspase-8 deficient), and Jurkat I 2.1 (FADD-deficient) were cultured in RPMI-1640 medium supplemented with 10% FCS, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). Human colon carcinoma HCT116 cell lines, wild-type HCT116-p53+/+ and its p53 null derivative HCT116-p53−/− (a generous gift from Dr B. Vogelstein, Johns Hopkins University, Baltimore, MD) were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). Osteosarcoma Saos-2 cells were grown as previously described (10).

**Generation of Enucleated Cells (Cytoplasts)**

P388 cells (2 × 10⁷/mL) were cultured in the presence of 10 μg/mL camptothecin B and 20 U/mL of DNase for 45 min at 37°C following procedures described previously (11), with minor modifications. The cell suspension was layered onto a discontinuous Ficoll density gradient (2 mL of 25%, 2 mL of 17%, 0.5 mL of 16%, 0.5 mL of 15%, and 2 mL of 12.5% Ficoll in complete medium containing 7.5 μg/mL camptothecin B and 40 U/mL of DNase preequilibrated in ultracentrifuge tubes at 37°C in a CO₂ incubator for 24 h). Gradients containing cell suspensions were centrifuged in a prewarmed SW41 rotor (Beckman) at 25,000 rpm for 30 min at 30°C. Cytoplasts were collected from the interface between 15% and 17% Ficoll layers, washed in complete medium (supplemented with insulin transferrin and selenium), and cultured overnight at 37°C before treatment with 5 μmol/L lamellarin D for 14 h. Cells were then stained with TMRM or FITC-VAD.fmk prior to the cyttofluorometric analysis.

**Determination of Nuclear Apoptosis**

The frequency of hypoploid (sub-G₁) cells was assessed as described (12). DNA fragmentation was determined by agarose gel electrophoresis as already described (13).

**Caspase Activity Assay**

The activity of caspases was determined with specific prosubstrates (Caspase-Glo luminescent kits from Promega Corp.) in triplicate following the manufacturer’s instructions. Luminescence was recorded with a Lumicount luminometer (Packard Instrument Company).

**Immunoblot Analysis**

Whole cell lysates were prepared as described (12). Equal protein quantities (50 μg) were subjected to SDS-PAGE. Membranes were blocked in 5% powdered milk in TBS Tween 0.05% for 1 h at room temperature and then incubated with primary antibodies specific for poly(ADP-ribose) polymerase (PARP) 1/2 (1:500; Santa Cruz, H-250), Puma (1:1,000; Cell Signaling), Noxa (1:1,000; Pro-science), Bid
(1:250, Santa Cruz, C-20), Bad (1:1,000; Sigma), Bim (1:1,000; Sigma), Bax (1:500; Santa Cruz, N20), Bcl-2 (1:500; Santa Cruz, Ac21), Mcl-1 (1:5,000; Rockland), Bcl-xl (1:1,000; Sigma, 2H12), caspase-9 (1:500, Santa Cruz, H-83), cleaved caspase-3 (1:500; Cell signaling, 5A1), caspase-2 (1:500; Santa Cruz, H-145), caspase-8 (1:500; Santa Cruz, D-8), XIAP (1:500; Stressgen, 2F1), cIAP2 (1:500; Santa Cruz, H-85), survivin (1:250; Santa Cruz, D-8), Fas-L (1:500; Santa Cruz, C-178), Fas (1:500; Santa Cruz, A20), GADD45α (1:500; Santa Cruz, C-4), p21 (1:500; Santa Cruz, F-5), p53 (1:500; Santa Cruz, FL-393), and phospho-p53 (Ser 15; 1:1,000; Cell Signaling). Secondary horseradish peroxidase–conjugated antibodies (BioRAD) were used at 1:2,000 for 1 h at room temperature, and detection was carried out by enhanced chemiluminescence. Anti-actin (1:5,000; Sigma) or anti-G3PDH antibodies (1:2,000; Trevigen) were used for standardization of protein loading. For analysis of cytochrome c release, cytosolic fractions were prepared using a method described previously (14).

**Cytolfluorometric Analysis of Apoptosis**

To detect total activated caspases by flow cytometry, the cell-permeant fluorochrome FITC-VAD.fmk (Promega) was used as described (15). To evaluate mitochondrial transmembrane potential (Δψm), cells (5 × 105/mL) were incubated for 20 min at 37°C with TMRM (125 nmol/L in PBS; λem = 573 nm). Samples were stored on ice prior to cytfluorometric analysis on a FACScan cytfluorometer (Becton Dickinson). For flow cytometric analysis of Bax activation, we used a procedure described elsewhere (13).

**Immunofluorescence Microscopy of p53**
P388 cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.1% Triton X-100 for 10 min, and washed twice in PBS with 2% FCS before incubation with a rabbit anti-p53 antibody (1:100; Santa Cruz, sc-6243) on ice overnight. After three washes in PBS, the cells were incubated with AlexaFluor488–conjugated antirabbit antibody (1:500; Molecular Probes) for 1 h at room temperature. DAPI was used for fluorescence counterstaining of nuclei. All samples were viewed and photographed with a fluorescence microscope (DMLR, Leica).

**γH2AX Analysis**
The H2AX Phosphorylation Assay kit (Upstate) was used following the manufacturer’s instructions. After staining with the anti-phospho-histone H2AX–FITC conjugate, which recognizes H2AX phosphorylated at serine 139, or with normal mouse IgG conjugate as control, cells were counterstained with propidium iodide for analysis of cell cycle (14). Fluorescence was measured by flow cytometry as described (16).

**Cell-Free System**
Nuclei were purified from P388 cells and mitochondria from rat liver following a method previously described (11). Mitochondria (25 μg of protein) and/or nuclei (10 × 10⁶) were resuspended in 50 μL of reaction buffer containing 20 mmol/L Hepes (pH 7.4), 10 mmol/L KCl, 2 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L DTT, 250 mmol/L sucrose, 10 mmol/L succinate, 2 mmol/L ATP, 10 mmol/L creatine phosphate, 50 μg/mL creatine kinase, and protease inhibitor cocktail (Sigma). The reaction mix was exposed to drugs at 37°C for 3 h after which mitochondria and nuclei were removed by centrifugation at 12,500 × g for 10 min at 4°C before immunoblot analysis.
Results

Cell Cycle Distribution and Nuclear Apoptosis Induced by Lamellarin D in P388 Cells

We previously reported that lamellarin D triggered the commitment step of the mitochondrial apoptotic cascade in leukemia cells (2). At 1 or 5 μmol/L, concentrations compatible with an in vivo antitumor activity (2), lamellarin D provoked a time-dependent accumulation of P388 leukemia cells in sub-G₁ (Fig. 1B) and internucleosomal DNA fragmentation, a hallmark for apoptosis (Supplementary Fig. S1). Similar results were obtained in human Jurkat T cells (not shown). At lower doses (<0.5 μmol/L), we have previously shown that lamellarin D can perturb cell cycle distribution with a marked arrest of the cells in S and G₂-M phase, a phenomenon presumably attributable to its inhibition of topoisomerase I (3, 4). The G₂-M arrest induced by 0.2 μmol/L lamellarin D was not followed by any increase of cells in sub-G₁ (Fig. 1B) even after protracted exposure (<10% sub-G₁ after 96 hours of exposure). We next evaluated the effect of a proapoptotic concentration of lamellarin D on cell cycle (Fig. 1C). The appearance of cells in sub-G₁ was not preceded by a prominent cell cycle arrest, although a

Figure 2. Lamellarin D activates the intrinsic pathway of apoptosis. A, P388 cells were incubated for the indicated times with or without 5 μmol/L lamellarin D. Whole-cell lysates were subjected to SDS-PAGE, blotted, and probed with antibodies recognizing caspase-2, cleaved caspase-3, caspase-9, and caspase-8. Arrows, cleaved products. The same blot was stripped and rebotted for caspase-2 and caspase-8. Actin served as a loading control. B, effect of caspase inhibitors on lamellarin D-induced apoptosis. P388 cells were cultured in the presence of 5 μmol/L lamellarin D and various inhibitors of proteases, i.e., phenylmethylsulfonylfluoride (1 mmol/L), pepstatine (10 μmol/L), Z-VAD.fmk (50 μmol/L), Z-VDVAD.fmk (50 μmol/L), Z-IETD.fmk (50 μmol/L), Z-LEHD.fmk (50 μmol/L), and Z-DEVD.fmk (50 μmol/L). At 24 h posttreatment, Δψₘ and sub-G₁ were determined by flow cytometry. Data are means ± SD of three independent experiments. C, Bcl-2 overexpression delays lamellarin D-induced viability loss. The effect of lamellarin D (5 μmol/L) on viability of bcl-2– (white square) or neo-transfected (black square) 2B4a11 T-cell hybridoma cells was evaluated over time. At the indicated time points (2, 4, 8, 18, 24, and 48 h of lamellarin D exposure), the percentage of viable cells was determined by flow cytometry staining with propidium iodide. D, the small-molecule Bcl-2 inhibitor HA14-1 acts with lamellarin D to increase mitochondria-dependent apoptosis. P388 cells were treated with lamellarin D (at indicated concentrations) and/or 10 μmol/L HA14-1 for 20 h, after which the percentage of cells displaying a loss of Δψₘ and sub-G₁ was determined by flow cytometry (means ± SD of three separate experiments in triplicate).
slight increase of cells in S phase was observed after 14 hours of incubation with 5 μmol/L lamellarin D. During apoptosis, activated effector caspases cleave various substrate proteins, including the nuclear enzyme PARP (17). Lamellarin D promoted PARP cleavage into a p85 fragment and this effect was inhibited by the pan-caspase inhibitor z-VAD.fmk (Fig. 1D). Similar protection was observed when apoptosis was detected by the percentage of cells in sub-G1 (2). Taken together, these results suggest that micromolar doses of lamellarin D induce caspase-mediated apoptosis of leukemia cells, independently of cell cycle arrest.

**Activation of the Intrinsic Caspase Cascade in Lamellarin D–Induced Cell Death**

To explore which caspases might be involved in lamellarin D–induced cell death, we analyzed caspases processing by immunoblotting. Lamellarin D induced cleavage of pro–caspase-9 as observed by the appearance of several bands of lower molecular weights (Fig. 2A). The time-dependent processing of caspase-9 was followed by the processing of caspase-3, occurring after 18 hours of treatment with lamellarin D. This result was consistent with the canonical concept of the intrinsic pathway placing caspase-3 downstream of caspase-9. In contrast, incubation of cells with lamellarin D failed to process caspase-8, the apical caspase of the extrinsic pathway, and caspase-2, the initiator caspase of apoptosis induced by cellular stress. We next correlated the processing of caspases with their activity (Supplementary Fig. S2). DEVDase (caspase-3/-7) and LEHDase (caspase-9) activities were detected in lamellarin D–treated P388 cells in a dose-dependent manner (Supplementary Fig. S2, left), confirming the results of immunoblot assays (Fig. 2A). We also detected a weak IETDase activity after lamellarin D exposure, but no VVDase (caspase-2) activity (Supplementary Fig. S2, right). The DEVDase and LEHDase activities were markedly inhibited in the presence of the corresponding inhibitors, showing the specificity of these results. This was not observed for IETDase activity, putting the validity its fluorimetric detection in our model in question. The pretreatment of cells with z-VAD.fmk was also effective against lamellarin D–induced caspase-9 and -3 activities, as it was against lamellarin D–induced nuclear apoptosis (Fig. 1D). Together, these results clearly implicate the involvement of the intrinsic caspase cascade in lamellarin D–triggered apoptosis.

**Lamellarin D Promotes Apoptosis through Activation of Mitochondria**

To determine the molecular orders between lamellarin D–mediated apoptotic events, P388 cells were pretreated with several caspase inhibitors, and apoptosis was assessed using the determination of Δψm and cells in sub-G1 (Fig. 2B). z-DEVD.fmk, z-LEHD.fmk, and the pan caspase inhibitor z-VAD.fmk largely suppressed lamellarin D–induced sub-G1, confirming that both caspase-3 and caspase-9 are functionally involved in lamellarin D–mediated apoptosis. Under the same conditions, none of the caspase inhibitors succeeded to inhibit the lamellarin D–induced drop in Δψm (Fig. 2B), nor did the pan–caspase inhibitor z-VAD.fmk inhibit the release of cytochrome c from the mitochondria (Supplementary Fig. S3). These data indicate that in lamellarin D–induced apoptosis, the caspase cascade (caspase-9 then caspase-3) operates downstream of mitochondria. Lamellarin D–induced apoptosis was not inhibited by the broad-spectrum aspartyl and serine protease inhibitors pestatin and phenylmethylsulfonylfluoride, suggesting that only caspases were involved in lamellarin D–mediated apoptosis. Experiments were done to explore the role of Bcl-2, a prototypically suppressor of the mitochondrial intrinsic pathway of apoptosis. 2B411 hybridoma T cells stably transfected with the bcl-2 gene or a neo control vector were treated with lamellarin D followed by determination of viability. As shown in Fig. 2C, loss of viability was significantly delayed by overexpression of Bcl-2, at least for the first 18 hours of incubation. At later time points, the protective effect of Bcl-2 was lost (Fig. 2C). We next determined the
ability of a small molecule Bcl-2 inhibitor, HA14-1, to reverse the effects of Bcl-2 on apoptosis induced by lamellarin D. P388 cells synergistically incubated with 10 μmol/L HA14-1 and lamellarin D for 24 hours had higher levels of cells in sub-G1 (Fig. 2D, rings) and a more pronounced drop in Δψm (Fig. 2D, bars) than those treated with lamellarin D alone. Altogether, these data support the contention that lamellarin D triggers the intrinsic, mitochondria-mediated pathway of apoptosis, which can be modulated by the Bcl-2 protein.

**The Fas-Dependent Extrinsic Pathway Is Not Required for Lamellarin D–Induced Apoptosis**

Several anticancer drugs activate both the intrinsic as well as the extrinsic pathway of apoptosis, particularly in leukemia cells (18). Therefore, we tested whether the apoptotic response triggered by lamellarin D also involves the activation of the extrinsic, death receptor–mediated, pathway. We first evaluated the expression of Fas and FasL after lamellarin D treatment. Time-course immunoblot analysis revealed that both Fas and FasL protein levels were not elevated after lamellarin D treatment (Fig. 3A). However, anticancer drugs can activate the extrinsic pathway independently of any change in Fas/FasL expression by promoting the intracellular recruitment of Fas-associated protein with death domain (FADD) to Fas and thereby favor caspase-8 activation (19). Thus, we used human Jurkat T leukemia cells lacking the caspase-8 adaptor protein FADD (FADD−/−) and Jurkat cells deficient of caspase 8 (caspase-8−/−) to determine whether the Fas pathway was required for lamellarin D–induced apoptosis. Lamellarin D promoted nuclear apoptosis in FADD−/− cells as well as in caspase-8−/− cells (Fig. 3B). In contrast, apoptosis was not induced by the Fas agonistic monoclonal antibody CH-11 used as control (Fig. 3B). There was no significant difference in the kinetics or dose response of apoptotic death induction by lamellarin D when comparing FADD−/− cells, caspase-8−/− cells, and wild-type Jurkat cells (data not shown). In addition, Δψm dissipation and mitochondrial cytochrome c release were observed in FADD−/− cells and caspase-8−/− cells after treatment with lamellarin D (Fig. 3C). These results indicate that the Fas/FasL death receptor pathway is not critical for apoptosis by lamellarin D.

**Lamellarin D Downregulates Expression of the Antiapoptotic Proteins Bcl-2 and cIAP2, and Activates the Proapoptotic Protein Bax in P388 Cells**

Because Bcl-2 protein is a critical regulator of lamellarin D–induced apoptosis (Fig. 2C and D), we investigated the expression of Bcl-2 family members at 2, 4, 8, and 18 hours of lamellarin D exposure (i.e., before completion of nuclear apoptosis). Figure 4A shows that the levels of the proapoptotic proteins Puma, Noxa, Bax, Bid, Bad, Bim, and Bcl-xL were not modified after treatment with lamellarin D, excluding a role of these proteins in lamellarin D–induced apoptosis. Although no upregulation of Bax was found (Fig. 4A), we observed that
lamellarin D led to significant Bax activation in a time- and dose-dependent manner (Fig. 4B). Lamellarin D induced a time-dependent reduction in the steady-state level of Bcl-2 beginning within 8 hours of treatment (Fig. 4A). A band corresponding to the full-length Bcl-2 disappeared, but no cleaved fragments could be detected. The degradation of Bcl-2 was caspase dependent because pretreatment of P388 cells with z-VAD.fmk maintained a high level of Bcl-2 expression (Fig. 4A). Conversely, lamellarin D did not modulate the expression of the other Bcl-2 family members, Bcl-xl or Mcl-1. These results suggest that lamellarin D shifts the balance of Bcl-2–related antiapoptotic and proapoptotic proteins to favor apoptotic demise.

The inhibitor of apoptosis protein (IAP) family members XIAP, cIAP1, cIAP2, and survivin are antiapoptotic proteins that inhibit caspases directly (20). These IAPs are expressed at high levels in P388 cells (Fig. 4C). Notably, lamellarin D-induced caspase-9 and caspase-3 activation correlated with a reduction in the level of cIAP2, detected already 4 hours post-lamellarin D exposure (Fig. 4C). The level of other IAPs remained unchanged (Fig. 4C).

DNA Damage following Lamellarin D Exposure

Topoisomerase I inhibitors like camptothecin are known to cause DNA damage, involving the formation of DNA double-strand breaks that trigger apoptosis (for review, see ref. 21). We used flow cytometry to detect the phosphorylated form of histone H2AX (γH2AX), which is a good indicator of double-strand DNA breaks in chromatin. Cells were cotained with propidium iodide for cell cycle analysis to correlate expression of γH2AX with cell cycle position (Fig. 5A). P388 cells exposed to lamellarin D displayed a significant increase in γH2AX fluorescence after a short incubation time (within 15 minutes) with a high response at 60 minutes (Fig. 5A). Nearly identical changes in DNA damage

Figure 5. Lamellarin D–induced DNA damage and p53 expression depend on the inhibition of nuclear topoisomerase I. A, multiparameter distribution representing expression of γH2AX versus DNA content of wild-type P388 cells (top) or topoisomerase I mutated P388 cells, P388 CPT5 (bottom), kept untreated (control; Co.), treated with 5 μmol/L lamellarin D for 15 or 60 min, or treated with the well-known DNA topoisomerase I inhibitor camptothecin (5 μmol/L) for 60 min. Based on difference in DNA content, G1, S, and G2-M cell populations were defined. Numbers in percentage represent the γH2AX positive cells gated. Data are representative of three independent experiments. B, immunoblot analysis of the DNA repair protein Rad51 in P388 and P388 CPT5 cells treated with 5 μmol/L lamellarin D for indicated times. Actin served as loading control. C, immunoblot analysis of p53 protein in P388 and P388 CPT5 cells treated with 5 μmol/L lamellarin D for indicated times. Actin served as loading control. D, left, immunofluorescence staining of p53 in untreated P388 cells (control; Co.) and in P388 cells 20 h post-lamellarin D treatment (5 μmol/L). Cells were stained with DAPI to display nuclear morphology. Original magnification ×40. Right, time course of p53 activation during lamellarin D treatment. P388 cells were treated with 5 μmol/L lamellarin D for 0 to 18 h and whole-cell lysates were subjected to immunoblotting with anti-phospho-Ser15 p53 antibody.
Mitochondria, DNA damage, and Lamellarin D

A

HCT116 Tp53+/−

% of Cells

Time (h)

0 4 24 48

HCT116 Tp53−

% of Cells

Time (h)

0 4 24 48

B

SAOS2

P53 null

C

D

FITC-VAD.fmk

E

Mitochondria

Mitochondria + nuclei

Co. Lam D Eto.
were observed for cells treated with the prototypic topoisomerase I inhibitor camptothecin (Fig. 5A). It is evident from these multiparameter analyses that the increase in γH2AX expression was most prominent for cells in S phase, a situation classically observed in response to topoisomerase I inhibitors (ref. 22 and Fig. 5A). In contrast, lamellarin D, as well as camptothecin, did not promote any nuclear damage in the topoisomerase I–mutated cells, P388 CPT5 (Fig. 5A), even upon long-term exposure (data not shown). Under these conditions, the double-strand breaks repair protein Rad51 was induced by lamellarin D at 8 hours exclusively in wild-type P388 cells (Fig. 5B). Collectively, these data indicate that lamellarin D may act as a classical nuclear topoisomerase I inhibitor to promote DNA damage and repair in P388 cells.

Role of p53 in Lamellarin D–Induced Apoptosis

The tumor suppressor p53 is generally activated in response to DNA damage in cancer cells. As shown in Fig. 5C, incubation of P388 cells with lamellarin D resulted in a time-dependent increase in the basal expression of the p53 protein not observed in P388 CPT5 cells. We also analyzed the subcellular localization of p53 by immunofluorescence microscopy (Fig. 5D). After lamellarin D treatment, p53 predominantly accumulated in the nucleus and no p53 immunoreactivity was detected in the cytoplasm or in mitochondria (Fig. 5D). This nuclear accumulation was more evident in apoptotic cells with condensed chromatin. Incubation of P388 cells with lamellarin D resulted in phosphorylation of p53 at Ser 15 (Fig. 5D, right). Nevertheless, the p53-inducible gene products Puma, Noxa, Bax (Fig. 4A), GADD45, and p21 (Supplementary Fig. 5) were not induced in P388 cells. Consistent with these data, we found that pifithrin-α, a chemical inhibitor of p53 transcriptional activity, was unable to impede apoptosis triggered by lamellarin D (data not shown). To rule out a transcription-independent proapoptotic function of p53, we tested the effect of lamellarin D on cancer cell lines possessing different p53 status (Fig. 6A and B). The wild-type HCT116 p53+/− colon carcinoma cells, as well as the HCT116 p53−/− cells, having a disrupted p53 gene due to homologous recombination, both displayed a reduction in mitochondrial Δψm and an increased number of cells in sub-G1 upon lamellarin D exposure (Fig. 6A). Although not statistically significant, the kinetic and extent of sub-G1 were more pronounced in p53+/− cells, suggesting that a functional p53 pathway might participate in lamellarin D–mediated apoptosis in HCT116 cells. The p53-null SAOS2 cells, although resistant to the induction of apoptosis by camptothecin, were sensitive to lamellarin D–induced apoptosis (Fig. 6B). Altogether these results indicate that intact p53 is not necessary for the apoptotic response triggered by lamellarin D.

We next examined the possibility that protein synthesis could be required for apoptosis induced by lamellarin D. Inhibition of protein synthesis by cycloheximide did not affect the appearance of nuclear and mitochondrial signs of apoptosis upon lamellarin D exposure, whereas a significant inhibition was observed for the dexamethasone-induced apoptosis, used as control (Fig. 6C). Finally, we studied the requirement of cell nucleus in lamellarin D–induced apoptosis. Upon lamellarin D treatment, both intact and enucleated P388 cells (cytoplasts) displayed a loss of mitochondrial Δψm to the same extent (Fig. 6D, left). Moreover, lamellarin D also triggered caspase activity irrespective of the presence of nucleus (Fig. 6D, right). To further document that the cell nucleus was dispensable for lamellarin D–induced apoptosis, we did a cell-free system experiment consisting of purified mitochondria combined with isolated P388 cell nuclei. Consistent with previous data (2), lamellarin D directly stimulated cytochrome c release from isolated mitochondria in a dose-dependent manner, even in the absence of nuclei (Fig. 6E). As a control, the DNA degrading drug etoposide (10 μmol/L) needed the presence of nuclei to promote mitochondrial release of cytochrome c in this reconstituted cell-free system. Together, these results show that apoptosis induced by lamellarin D is strictly dependent on mitochondria and can occur without protein synthesis and nuclear involvement.

Discussion

The discovery of the antitumor activity of lamellarin D encouraged molecular studies aimed at deciphering how this natural product and its synthetic derivatives were able to inhibit the growth of different cancer cells. This growth inhibition involves both decreased cell division (4) and

Figure 6. p53-dependent and nuclear-dependent signaling pathways are not required for lamellarin D–induced apoptosis. A, p53+/+ and p53−/− HCT116 colon carcinoma cells were incubated with lamellarin D (1 or 5 μmol/L) for the indicated times before determination of cells in sub-G1. Camptothecin (5 μmol/L 24 h) was used as a control for p53-dependent apoptosis. Results (mean ± SD) are representative of at least five independent experiments. B, The SAOS2 osteosarcoma cell line (p53 null) was incubated with lamellarin D for 24 h before determination of sub-G1 and Δψm. Camptothecin (5 μmol/L 24 h) was used as a control for p53-dependent apoptosis. Results (mean ± SD) are representative of three independent experiments. C, protein synthesis is dispensable for lamellarin D–triggered apoptosis. P388 cells were incubated with either 5 μmol/L lamellarin D or 1 μmol/L dexamethasone (DEX) alone or in the presence of 1 μmol/L cycloheximide (CHX). After 4 h of culture, changes in mitochondrial membrane potential (Δψm) were assessed by the use of TMRM. Alternatively, after 12 h of lamellarin D treatment, the percentage of cells in sub-G1 was determined. Results (mean ± SD) are representative of three independent experiments. D, comparison of enucleated P388 cells (cytoplasts) and intact P388 cells in response to lamellarin D. Cytoplasts or intact P388 cells were treated with lamellarin D at indicated concentrations for 6 h. Left, mitochondrial membrane potential was determined by flow cytometry using TMRM and the values are relative mean fluorescence intensity ± SD of three independent experiments. Right, detection of pan-caspase activity with FITC-VAD.fmk. Cytoplasts and P388 control cells were incubated for 15 h with or without 5 μmol/L lamellarin D, and caspase activity was assessed by flow cytometry. Results are representative of two independent experiments. E, lamellarin D induces cytochrome c release in a cell-free system. Upper box, mitochondria isolated from P388 cells (0.5 mg/mL final concentration) were incubated for 90 min at 37°C in the absence (control; Co.) or presence of lamellarin D (0.5, 2, and 5 μmol/L) or 10 μmol/L etoposide (Eto.). Lower box, conditions as in upper box, except that nuclei (2 × 105/mL) isolated from P388 cells were added to the samples. At the end of the incubation time the reaction mixture was centrifuged and supernatants were immunoblotted for the detection of cytochrome c release.
induction of apoptosis (2), and it seems that these two antitumor actions are dose dependent. When used at very low doses (nanomolar range), lamellarin D did not kill cancer cells but induced an accumulation of cells in S and G2-M phases, dependent on its inhibition of topoisomerase I (Fig. 1B and ref. 2). Conversely, at higher doses of lamellarin D (micromolar range compatible with the xenograft assays; ref. 23), cancer cells displayed a potent apoptotic response without major effects on cell cycle (2). In cancer, the therapeutic goal is to trigger tumor-selective cell death (24), and the response of tumors to therapy mainly depends on their ability to undergo cell death (24). Nowadays, the role of apoptosis in the cytotoxicity of anticancer drugs has become clearer (6). Different classes of anticancer drugs exert their cytotoxicities through a variety of primary molecular targets to ultimately complete cell death via common apoptotic signaling pathways (24). Our data clearly indicate that lamellarin D activates the intrinsic, mitochondria-dependent, apoptotic pathway in cancer cells. This is based on several pieces of evidence: (a) proteolytic activation of caspase-9; (b) induction of caspase-independent cytochrome c release; (c) modulation of the Bcl-2 protein, which influences the level of apoptosis induced by lamellarin D; and (d) the occurrence of lamellarin D–induced cell death independently of the extrinsic death receptor–mediated pathway. We considered the possibility that lamellarin D, by reducing the expression of antiapoptotic proteins (e.g., Bcl-2), favors apoptosis through amplification of the mitochondria-dependent pathway. We observed a reduced expression of the Bcl-2 protein associated with a conformational activation of Bax. These Bcl-2 family proteins normally function to control the mitochondrial release of cytochrome c (25). However, our results show a reduction in Bcl-2 level long after the onset of cytochrome c release (2). Based on these results, we can assume that caspase-mediated cleavage of Bcl-2 represents a feedback loop for the amplification of mitochondrial cytochrome c release during lamellarin D–induced apoptosis. In addition to Bcl-2 family proteins, we also noticed that lamellarin D reduced levels of the antiapoptotic protein, cIAP2. IAP family members are generally overexpressed in cancer and their downregulation contributes to an increased sensitivity to chemotherapeutic drugs. cIAP2 inhibits caspase-9 and caspase-3, thereby blocking the intrinsic caspase cascade downstream of mitochondria. It is conceivable that the capacity of lamellarin D to activate the intrinsic pathway downstream of mitochondria was also amplified by its inhibitory effect on cIAP2. The mechanisms of cIAP2 reduction were independent of caspases (data not shown). cIAP2 is subjected to regulation by the second mitochondrial activator of caspase (Smac/Diablo), a protein that is released from mitochondria during apoptosis (20). Thus, it is possible that the reduced levels of cIAP2 might be a downstream consequence of mitochondrial membrane permeabilization, and hence the release of Smac/Diablo. Further studies should clarify the mechanisms responsible for cIAP2 reduction.

Most conventional chemotherapeutic drugs are nucleus-targeted (genotoxic) agents that have been designed to interact with DNA or DNA-associated proteins such as topoisomerases (26). As a true topoisomerase I inhibitor, lamellarin D induced a time-dependent DNA damage response assessed by staining for γ-H2AX and Rad51 in P388 cells. In response to lamellarin D–induced DNA damage, levels of activated, Ser15-phosphorylated p53 increased markedly. However, no activation of the p53 downstream targets GADD45, p21, Bax, Puma, and Noxa, was observed. One possible explanation is that P388 cells do not respond to p53-mediated apoptosis due to mutations in the p53 binding site, as it has been suggested (27). Transcription-independent activities of p53 are important for the apoptotic response (28). Thus, p53 can be translocated to mitochondria to cause cell death in response to DNA damage (for review see ref. 8). It is hardly probable, however, that the early mitochondrial permeability observed after lamellarin D exposure is a consequence of p53 translocation to mitochondria because there was no p53 translocation to mitochondria (Fig. 5D). Furthermore, in p53 mutated or null cells, mitochondria in themselves seem sufficient to trigger apoptosis upon lamellarin D exposure. Indeed, although we were able to detect a moderate dose-dependent increase of the proapoptotic effect of lamellarin D in wild-type p53-expressing cells when compared with p53−/− cells (Fig. 6A), this effect of p53 was limited and transient because no further increase in apoptosis was observed after 48 hours of treatment with lamellarin D. These results indicate that p53 is not critical for the completion of lamellarin D–induced apoptosis. Interestingly, this characteristic distinguishes lamellarin D from camptothecin, the prototypic topoisomerase I poison.

These results prompted us to further explore whether lamellarin D–induced DNA damage might generate other nuclear signaling pathways contributing to apoptosis. Using enucleated cells and cell-free system assays, our results show that when lamellarin D was targeted to mitochondria, cells underwent apoptosis. Hence, its nuclear targeting seemed dispensable. These results raise the possibility of “double hits” of lamellarin D, one directly on the mitochondria to trigger apoptosis and the other located in the cell nucleus, resulting in DNA damage, cell cycle arrest, and DNA repair. Like lamellarin D, betulinic acid is a mitochondria-targeting drug and an inhibitor of nuclear topoisomerase I (29). Moreover, betulinic acid triggers apoptosis by a direct effect on mitochondria, independent of its nuclear action (30). Thus, lamellarin D triggers an apoptotic pathway different from those previously identified for standard genotoxic chemotherapeutic drugs such as topoisomerase I inhibitors (26). Unlike camptothecin, lamellarin D exerts a second mode of action, bypassing the nucleus and directly triggering apoptosis via the mitochondria. In theory, direct targeting of the mitochondria may circumvent drug resistance caused by most of the apoptotic defects. Lamellarin D kills a variety of tumor cells including several leukemia and carcinoma cell lines (2, 4), suggesting that the mechanism of apoptosis is not cell type dependent. This broad antitumor activity may result from its mitochondrial targeting. As a result, mitochondriophilic agents, like lamellarin D, may prove to be highly efficient in killing...
cancer cells normally resistant to apoptosis (31). In recent years, mitochondria have emerged as a promising target for cancer therapy (32), and clinical evaluation of several drugs targeting mitochondria-related proteins of the Bcl-2 family (GX15-070, oblimersen sodium, ABT-737) are ongoing (33).

In summary, this article not only contributes to deciphering the apoptotic pathways activated by lamellarin D but also provides evidence that lamellarin D is still active even if the classical apoptotic pathways are blocked in cancer cells (e.g., when p53 is null/mutated, when topoisomerase 1 is mutated or Bcl-2 overexpressed). This study provides the basis for new strategies establishing mitochondria as a valuable target in cancer cells, reinforcing the potential interest of mitochondriophilic drugs for cancer therapy. Targeting mitochondria may confer a solid advantage against drug resistance in cancer cells, provided that a selectivity for tumor versus normal mitochondria can be delineated. It will be therefore essential to identify the molecular target(s) of lamellarin D in mitochondria. Studies are ongoing in our laboratories.

 Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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