Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines

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Abstract

Bovine lactoferricin (LfcinB) is a cationic, amphipathic peptide that is cytotoxic for human and rodent cancer cells. However, the mechanism by which LfcinB causes the death of cancer cells is not well understood. Here, we show that *in vitro* treatment with LfcinB rapidly induced apoptosis in several different human leukemia and carcinoma cell lines as determined by DNA fragmentation assays and phosphatidylserine headgroup inversion detected by Annexin V binding to the surface of cancer cells. Importantly, LfcinB treatment did not adversely affect the viability of untransformed human lymphocytes, fibroblasts, or endothelial cells. Studies with different LfcinB-derived peptide fragments revealed that the cytotoxic activity of LfcinB resided within the amino acid sequence FKCRRWQWRM. Treatment of Jurkat T leukemia cells with LfcinB resulted in the production of reactive oxygen species followed by caspase-2-induced dissipation of mitochondrial transmembrane potential and subsequent activation of caspase-9 and caspase-3. Selective inhibitors of caspase-2 (Z-VDVAD-FMK), caspase-9 (Z-LEHD-FMK), and caspase-3 (Z-DEVD-FMK) protected both leukemia and carcinoma cells from LfcinB-induced apoptosis. Conversely, a caspase-8 inhibitor (Z-IETD-FMK) had no effect, which argued against a role for caspase-8 and was consistent with the finding that death receptors were not involved in LfcinB-induced apoptosis. Furthermore, Jurkat T leukemia cells that overexpressed Bcl-2 were less sensitive to LfcinB-induced apoptosis, which was characterized by mitochondrial swelling and the release of cytochrome *c* from mitochondria into the cytosolic compartment. We conclude that LfcinB kills cancer cells by triggering the mitochondrial pathway of apoptosis at least in part through the generation of reactive oxygen species. [Mol Cancer Ther 2005;4(4):612–24]

Introduction

Bovine lactoferricin (LfcinB) is a peptide fragment produced by acid-pepsin hydrolysis of lactoferrin obtained from cow's milk (1). LfcinB, which consists of amino acid residues 17 to 41 proximal to the NH_2 terminus of bovine lactoferrin, is notable for its inability to bind iron and relatively high proportion and asymmetrical clustering of basic amino acid residues. Substantial levels of LfcinB are found in the human stomach following ingestion of bovine lactoferrin (2), implying that LfcinB is a natural breakdown product from the digestion of cow's milk. Although LfcinB has attracted considerable interest because of its well-established antimicrobial activity (3–5), recent evidence indicates that LfcinB also possesses potent *in vivo* activity against cancer cells (6–8). S.c. administration of LfcinB to mice 1 day after i.v. tumor inoculation results in a significant inhibition of lung and liver metastasis by L5178Y-ML25 murine lymphoma cells as well as lung metastasis by B16-BL6 murine melanoma cells (6). The same study showed an inhibitory effect by LfcinB on both tumor-induced angiogenesis and tumor growth in mice inoculated intradermally with B16-BL6 melanoma cells. In addition, intratumoral injections of LfcinB slow the growth of murine Meth A fibrosarcoma cells grown as s.c. tumors in mice (8). Furthermore, oral administration of LfcinB to rats that had been injected previously with azoxymethane to promote colon carcinogenesis results in an impressive 83% reduction in the incidence of colon adenocarcinomas (7). A similar effect was achieved by oral administration of intact bovine lactoferrin, which raises the intriguing possibility that LfcinB derived from dietary bovine lactoferrin may protect against colon carcinogenesis. Interestingly, consumption of milk and milk products has been linked recently to a reduced risk of colorectal cancer in humans (8) as well as reduced tumor growth in mice treated with 1,2-dimethylhydrazine to induce colon cancer (9).

Remarkably little is known about the mechanism by which LfcinB exerts its anticancer activity, although the available evidence favors a direct inhibitory effect of LfcinB on cancer cell growth and metastasis. A recent study indicates that *in vitro* exposure to LfcinB causes murine Meth A fibrosarcoma cells to lose membrane integrity and eventually lyse (10). In addition, LfcinB is a potent inducer of apoptosis in cultures of THP-1 human monocytic leukemia cells (11). In contrast, intact bovine lactoferrin is

Received 3/18/04; revised 1/26/05; accepted 2/7/05.

Grant support: Natural Sciences and Engineering Research Council of Canada; Dairy Farmers of Canada grants-in-aid; Cancer Research Training Program Studentship with funding from Dalhousie Cancer Research Program and Nova Scotia Health Research Foundation Studentship (J.S. Mader); and Natural Sciences and Engineering Research Council of Canada postgraduate scholarship (D.M. Conrad).

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unable to trigger apoptosis in THP-1 leukemia cells even at 10-fold higher concentrations than LfcinB, indicating that apoptosis-inducing activity is unique to LfcinB. However, it is not known whether LfcinB is an effective inducer of apoptosis in a broader range of human cancer cells, including more common cancers that are of epithelial origin. Moreover, the effect of LfcinB on the viability of untransformed human cells has not been assessed.

Apoptosis is a distinct form of cell death that plays important roles in embryonic development, immune system maturation and cytotoxic effector function, and carcinogenesis (12). Apoptotic cells are characterized by several unique features, including cell shrinkage, chromatin condensation, DNA fragmentation, cell surface expression of phosphatidylserine, and membrane blebbing. Two main pathways mediate the activation and subsequent execution of apoptosis. The extrinsic/death receptor pathway is triggered by ligand-induced aggregation of death receptors, such as Fas and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) DR4 and DR5 (13), whereas the intrinsic/mitochondria-dependent pathway is activated in response to radiation or cytotoxic drug-induced cellular stress (14). Caspases are aspartate-specific cysteine proteases that play critical roles in the initiation and execution of both pathways of apoptosis (15). Caspases are synthesized as inactive proenzymes that are subsequently activated by enzymatic cleavage after aspartic acid residues. Caspase-8 and caspase-10 are initiator caspases for the death receptor pathway of apoptosis (16, 17), whereas caspase-2 and caspase-9 are initiator caspases for the mitochondria-dependent pathway of apoptosis (18, 19). Pro-caspase-9 is recruited and activated by the apoptosome, which consists of oligomerized cytosolic Apaf-1 and cytochrome *c* released from mitochondria in response to diminished mitochondrial membrane integrity (20). Initiator caspases are characterized by long prodomains that allow interactions with specific adaptor proteins, bringing initiator caspases into close proximity with each other and promoting caspase activation via mutual cleavage events (21). Activation of initiator caspases leads to the direct or indirect activation of executioner caspases, including caspase-3, caspase-6, and caspase-7 (22). Activation of executioner caspases and the subsequent generation of feedback amplification loops lead to the proteolysis of cytoplasmic and nuclear polypeptide substrates, including major structural elements of the cytoplasm and nucleus, proteins involved in DNA repair, and several different protein kinases, which ultimately results in cell death (15). In addition, at least one endonuclease is activated directly by executioner caspases (23). Oxidative stress created by an imbalance between reactive oxygen species (ROS) and cellular antioxidants has been implicated in the induction of both death receptor– and mitochondria-initiated apoptosis (24, 25). Interestingly, ROS are involved in LfcinB-induced apoptosis of THP-1 leukemia cells (11), although it is not known whether the production of ROS is a proximal or distal event in the apoptotic process.

The goals of the current study are 4-fold. First, it is important for possible future clinical applications to determine whether the apoptosis-inducing activity of LfcinB extends to human cancer cells of epithelial as well as hematopoietic origin, without causing harm to normal human cells. In this regard, toxicity by conventional chemotherapeutic agents remains a major challenge in the treatment of human malignancies (26). The second objective is to examine shorter derivatives of LfcinB for apoptosis-inducing activity because smaller peptide fragments would be more easily synthesized in large quantities for therapeutic use. The third goal is to determine the role of death receptor(s) and mitochondria as well as caspases in LfcinB-induced apoptosis of human cancer cells. A detailed understanding of the mechanism of LfcinB-induced killing of human neoplasms may suggest possible synergic interactions with other anticancer agents as has already been established for recombinant TRAIL and different chemotherapeutic drugs in several tumor cell lines (27, 28). Finally, we wish to confirm the contribution of ROS to LfcinB-induced apoptosis in cultures of human cancer cells and determine whether ROS production is involved in the initiation or execution phase of apoptosis.

Materials and Methods

Cell Lines and Reagents

CCRF-CEM cells were kindly provided by Dr. W. Gati (University of Alberta, Alberta, Edmonton, Canada), whereas MDA-MB-435 cells were a generous gift from Dr. J. Mackey (University of Alberta). Bcl-2-overexpressing Jurkat T leukemia cells and vector-only transfectants (29) were generously contributed by Dr. C. Bleackley (University of Alberta). All other tumor cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were maintained at 37°C in a 5% or 10% CO_2 humidified atmosphere in RPMI 1640 or DMEM (Sigma-Aldrich Canada, Oakville, Ontario, Canada), respectively, supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin, 2 mmol/L L-glutamine, 5 mmol/L HEPES buffer (pH 7.4), and 5% or 10% heat-inactivated FCS (Invitrogen, Burlington, Ontario, Canada) as appropriate for each cell line. Stock flasks were passaged twice weekly or as required to maintain optimal cell growth. Normal human T cells, umbilical vein endothelial cells, and fibroblast cells were generous gifts from Drs. J. Marshall, A. Issekutz, and T. Peterson (Dalhousie University, Halifax, Nova Scotia, Canada), respectively. All primary cell cultures were maintained at 37°C in a 10% CO_2 humidified atmosphere. LfcinB (amino acid sequence: FKCRRWQWRMKKLGAPSITCVRRAF) and its 10-mer (amino acid sequence: FKCRRWQWRM) and 6-mer (amino acid sequence: RRWQWR) derivatives were synthesized in linear form by Sigma Genosys (The Woodlands, TX) with a purity of >95%. For some preliminary experiments, we used pepsin-generated LfcinB that was provided generously by Morinaga Milk Industry Co. (Zama, Japan). Lyophilized peptides were dissolved in serum-free RPMI

1640 and aliquots were stored at −70°C. All experiments with LfcinB and its derivatives were done with medium containing 0.5% FCS, because these peptides exhibit optimal cytotoxic activity at lower serum concentrations (11). *N*-acetyl-L-cysteine (NAC) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Canada. Glutathione, caspase inhibitors (Z-VAD-FMK, Z-VDVAD-FMK, Z-LEHD-FMK, Z-IETD-FMK, and Z-DEVD-FMK), and chromogenic caspase substrates (Ac-IETD-pNA, Ac-VDVAD-pNA, Ac-LEHD-pNA, and Ac-DEVD-pNA) were from EMD Biosciences, Inc. (San Diego, CA). Dihydroethidium and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes (Eugene, OR). Mouse anti-human Fas and mouse anti-human Fas ligand monoclonal antibodies (mAb) were from BD PharMingen (Mississauga, Ontario, Canada). FITC-conjugated goat anti-mouse IgG (H + L) was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). Phycoerythrin (PE)–conjugated mouse anti-human TRAIL (RIK-2) mAb, PE-conjugated mouse anti-human DR4 mAb, PE-conjugated mouse anti-human DR5 mAb, and mouse IgG were from eBioscience (San Diego, CA). Rabbit anti-human caspase-9 antibody, rabbit anti-human caspase-3 antibody, and mouse anti-human caspase-8 mAb were from Cell Signaling Technology (Beverly, MA). Rabbit anti-human caspase-2 antibody was from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada) and mouse anti–cytochrome *c* mAb was from Upstate Biotechnology (Charlottesville, VA). Goat anti-mouse IgG-horseradish peroxidase and goat anti-rabbit IgG-horseradish peroxidase were from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA Fragmentation Assays

The JAM assay was used to measure DNA fragmentation in cells undergoing apoptosis (30). Briefly, cells were labeled with tritiated thymidine (ICN Biomedicals, Irvine, CA; 5 μCi/mL of cells) for 4 hours at 37°C, washed twice with medium, and resuspended in fresh medium containing 0.5% FCS. Radiolabeled cells (2.5×10^5 cells/mL) were added in quadruplicate to 96-well flat-bottomed tissue culture plates (Sarstedt, Inc., St. Laurent, Quebec, Canada) in the absence or presence of the desired concentrations of LfcinB. Following 18-hour incubation at 37°C in a 5% CO_2 humidified atmosphere, DNA was harvested onto glass fiber filter mats using a multiple sample harvester (Skatron Instruments, Sterling, VA). Radioactivity was measured by liquid scintillation counting. Percentage of specific apoptosis was calculated as follows: % Specific apoptosis = $(S_{cpm} - E_{cpm}) / S_{cpm} \times 100$, where S is DNA retained from control cells and E is DNA retained from LfcinB-treated cells. Alternatively, DNA was isolated from control or LfcinB-treated cells using a kit purchased from Qiagen, Inc. (Mississauga, Ontario, Canada) and DNA fragmentation was visualized by gel electrophoresis as described previously (31).

MTT Assay

As an alternative to the JAM assay, changes in cell viability were also assessed by MTT assay (32). Cells were resuspended in fresh medium containing 0.5% FCS and added in quadruplicate to 96-well flat-bottomed tissue culture plates. After 15-hour culture in the absence or presence of the desired concentrations of LfcinB, MTT was added to each well at a final concentration of 500 μg/mL and the plates were incubated for an additional 3 hours. The medium was then removed and DMSO (0.1 mL) was added to each well to solubilize the cells. Spectrometric absorbance was measured at 492 nm.

Death Receptor/Ligand Expression

Cells were incubated for 45 minutes at 4°C with 10 μg/mL primary mAb (anti-Fas, anti–Fas ligand, PE-anti-TRAIL, PE-anti-DR4, or PE-anti-DR5) in immunofluorescence buffer (1% bovine serum albumin, 0.2% sodium azide in PBS). Cells were then washed thrice with immunofluorescence buffer and, if appropriate, incubated for an additional 45 minutes at 4°C with FITC-anti-mouse IgG to detect cell-bound anti-Fas or anti–Fas ligand mAb. After three additional washes with immunofluorescence buffer, cells were resuspended in 1% paraformaldehyde in PBS and analyzed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Determination of Mitochondrial Transmembrane Potential

Flow cytometric analysis of cells stained with $DiOC_6$ was used to measure changes in mitochondrial transmembrane potential (33). $DiOC_6$ was stored at 4°C as a 1 mmol/L stock in DMSO. Cells were exposed to medium alone (containing 0.5% FCS) or LfcinB (200 μg/mL) for 1, 2, and 4 hours in wells of a 24-well flat-bottomed tissue culture plate at 37°C in a 5% CO_2 humidified atmosphere. $DiOC_6$ was then added to untreated or LfcinB-treated cells at a final concentration of 40 nmol/L, and 30 minutes later, cells were analyzed by flow cytometry.

Annexin V Labeling of Apoptotic Cells

Annexin V staining was used to measure phosphatidylserine headgroup externalization on cells undergoing apoptosis (34) according to the instructions provided by the manufacturer of the Annexin V staining kit (Roche Diagnostics, Laval, Quebec, Canada). Briefly, 1×10^6 cells were exposed to medium alone (containing 0.5% FCS) or LfcinB (200 μg/mL) for the desired length of time at 37°C in a 5% CO_2 humidified atmosphere, washed, resuspended in 0.1 mL staining solution (2% Annexin V-FITC and 2% propidium iodide-PE by volume in HEPES buffer), and incubated for an additional 10 to 15 minutes. Cells were then analyzed by flow cytometry.

Measurement of ROS

Flow cytometry and dihydroethidium were used to measure the production of ROS during apoptosis (35). Briefly, cells were exposed to medium alone (containing 0.5% FCS) or LfcinB (200 μg/mL) for the desired length of time in wells of a 24-well flat-bottomed tissue culture plate at 37°C in a 5% CO_2 humidified atmosphere. Dihydroethidium was then added to cells at a final concentration of 2.5 μmol/L, and 20 minutes later, cells were analyzed by flow cytometry.

Immunoblotting

Cells (5×10^6) were exposed to LfcinB (200 μg/mL) for 2 hours at 37°C in a 5% CO_2 humidified atmosphere and then washed in PBS. For caspase detection, the cell pellet

was resuspended in 0.1 mL ice-cold CHAPS lysis buffer [50 mmol/L PIPES/NaOH (pH 6.5), 2 mmol/L EDTA, 5 mmol/L DTT, 20 μg/mL leupeptin, 10 μg/mL pepstatin, 10 μg/mL aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1% CHAPS] and placed on ice for 1 hour with vortexing every 10 minutes. Samples were then clarified by centrifugation at 10,000 × *g* for 10 minutes at 4°C and the supernatant was collected. For cytochrome *c* detection, the cell pellet was resuspended in 0.1 mL ice-cold digitonin lysis buffer (75 mmol/L NaCl, 1 mmol/L NaH_2PO_4, 8 mmol/L Na_2HPO_4, 250 mmol/L sucrose, 2 mmol/L sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 190 μg/mL digitonin) and incubated on ice for 15 minutes to allow digitonin to selectively permeabilize the plasma membrane. Cells were then pelleted by centrifugation at 15,000 × *g* for 5 minutes at 4°C and the supernatant was collected. Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). Samples were boiled in SDS sample buffer and total protein (20 μg) was loaded into each well of a 12% (for caspase detection) or 15% (for cytochrome *c* detection) SDS-polyacrylamide gel for separation by electrophoresis. Protein bands were transferred onto nitrocellulose membranes. The resulting blots were blocked overnight with PBS-Tween 20 [0.25 mol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.2% Tween 20 in PBS] containing 5% powdered skim milk and then probed overnight with the desired primary antibody at a 1:500 dilution. Blots were then washed with PBS-Tween 20 and probed for 1 hour with horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG (1:1,000 dilution) as appropriate. Following additional washes with PBS-Tween 20, the protein bands were visualized using an enhanced chemiluminescence detection system (Bio-Rad Laboratories).

Caspase Activity Assay

Caspase activity in cell lysates was measured using a chromogenic assay developed from a protocol described by Talanian et al. (36). Briefly, 1×10^6 cells were resuspended in medium containing 0.5% FCS and incubated with or without LfcinB (200 μg/mL) for 2 hours at 37°C in a 5% CO_2 humidified atmosphere. Cells were then pelleted and resuspended in ice-cold lysis buffer (20 mmol/L HEPES, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 0.25% Triton X-100) followed by intermittent vortexing for 5 minutes at 4°C. Lysates were cleared by centrifugation at 4°C and aliquots were added in quadruplicate to wells of a 96-well flat-bottomed plate with or without the appropriate chromogenic caspase substrate (Ac-IETD-pNA, Ac-VDVAD-pNA, Ac-LEHD-pNA, or Ac-DEVD-pNA) at a final concentration of 200 μmol/L. Plates were incubated at 37°C and caspase activity in individual samples was determined by measuring absorbance at 405 nm every 15 minutes for 2 hours with a microplate autoreader (Bio-tek Instruments, Winooski, VT). Relative caspase activity was determined by subtracting background readings obtained in the absence of cell lysates. An absorbance of 0.001 was arbitrarily defined as 1 unit of caspase activity.

Electron Microscopy

Electron microscopy was done in the Electron Microscope Facility of the Faculty of Medicine, Dalhousie University, using standard protocols (37). A Philips EM300 transmission electron microscope operating at 60 kV was used to visualize Jurkat T leukemia cells that had been cultured in the absence or presence of 200 μg/mL LfcinB for 2 hours.

Results

LfcinB Causes Apoptosis in Human Leukemia and Carcinoma Cells without Affecting the Viability of Untransformed Cells

Figure 1A shows that an 18-hour exposure to synthetic LfcinB (200 μg/mL) was cytotoxic for different human leukemia and carcinoma cell lines as determined using the JAM assay. A similar cytotoxic effect was obtained when Jurkat T leukemia cells or MCF-7 breast carcinoma cells were treated with 200 μg/mL pepsin-generated LfcinB (86 ± 2% and 43 ± 3% cell death, respectively). These data strongly suggested that LfcinB caused tumor cells to die by apoptosis because the JAM assay measures DNA fragmentation (30). In contrast, LfcinB was not cytotoxic to primary cultures of normal human T lymphocytes (resting and mitogen-activated), fibroblasts, or endothelial cells. A MTT assay (32) was used to determine the effect of LfcinB on the viability of untransformed cells because their slow rate of proliferation did not allow for the high level of tritiated thymidine incorporation that is required to measure cell death by JAM assay. LfcinB treatment triggered DNA fragmentation in T leukemia cells (Jurkat), breast cancer cells (MCF-7), and colon cancer cells (Colo-35) in a dose-dependent fashion, with a cytotoxic effect being evident at LfcinB concentrations as low as 25 μg/mL (Fig. 1B). A time course analysis revealed that LfcinB-induced DNA fragmentation in Jurkat T leukemia cells was detectable within 2 hours of treatment with 200 μg/mL LfcinB and became maximal by the 8-hour time point (Fig. 1C). LfcinB-induced DNA fragmentation in MDA-MB-435 breast carcinoma cells was slightly delayed in comparison with Jurkat T leukemia cells, although substantial cell death was evident by the 16-hour time point. Jurkat T leukemia cells were chosen for use in most of the following experiments because of the sensitivity that these transformed T cells exhibited to the cytotoxic activity of LfcinB.

To confirm that LfcinB caused apoptosis in human cancer cells, we treated Jurkat T leukemia cells with 200 μg/mL LfcinB for 4 hours and isolated DNA, which was subsequently electrophoresed across an agarose gel and visualized by ethidium bromide staining. Figure 2A shows that only intact high molecular weight DNA was present in the lane containing DNA from untreated control cells (*lane 2*), whereas nucleosomal-sized DNA fragments were present in the lane containing DNA from LfcinB-treated cells (*lane 3*). Moreover, the DNA laddering effect induced by LfcinB was comparable with that induced by etoposide (*lane 4*), which is a topoisomerase II

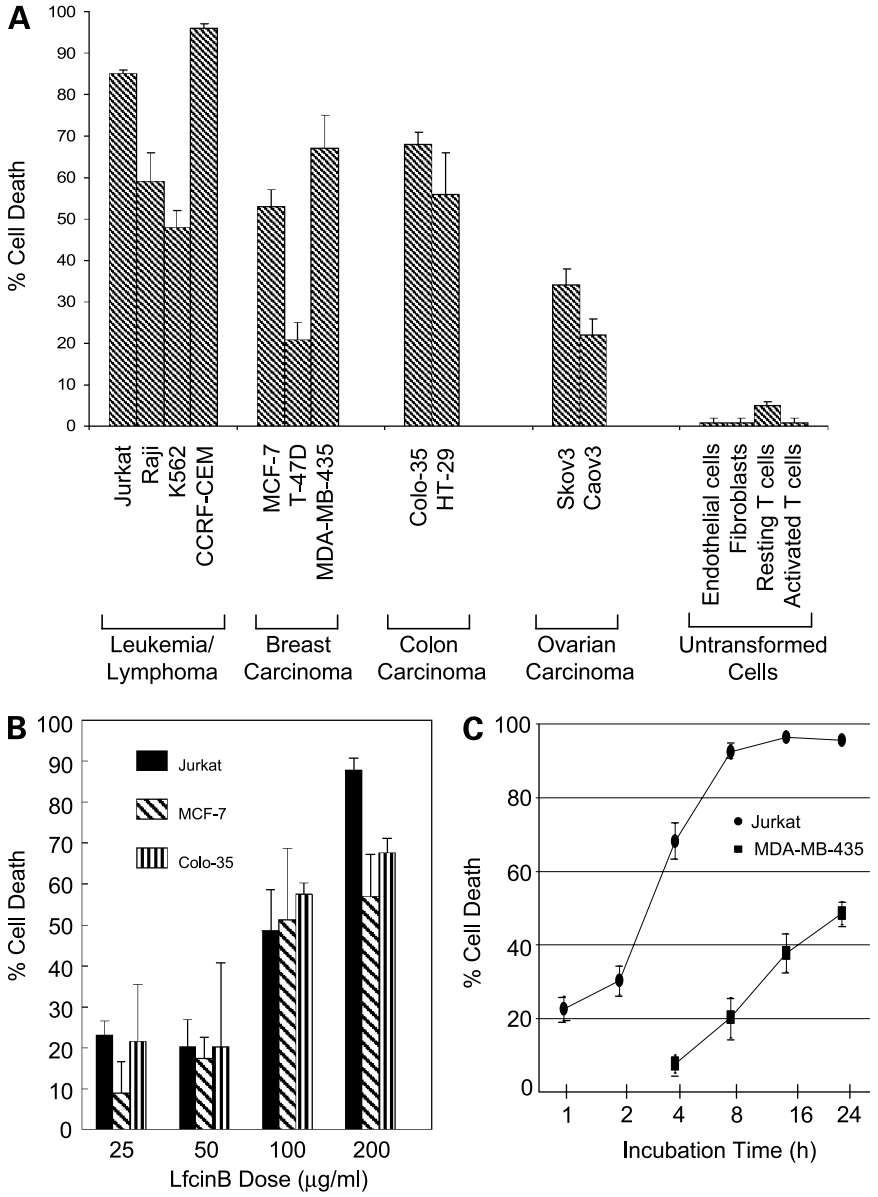

Figure 1. LfcinB is cytotoxic to human cancer cells but not to untransformed cells and mediates its effects in a dose- and time-dependent fashion. **A,** human cancer cell lines of hematopoeitic and epithelial (breast, colon, and ovary) origin as well as normal human T cells (resting and stimulated with 5 μg/mL concanavalin A), endothelial cells, and fibroblasts were treated with 200 μg/mL LfcinB for 18 h. Cytotoxicity against cancer cells was determined by JAM assay, whereas cytotoxicity against normal cells was determined by MTT assay, because the slow rate of proliferation by normal cells resulted in insufficient tritiated thymidine incorporation for analysis of cell death by JAM assay. **B,** Jurkat T leukemia cells, MCF-7 breast cancer cells, or Colo-35 colon cancer cells were treated with the indicated concentrations of LfcinB for 18 h. Cytotoxicity was then determined by JAM assay. **C,** Jurkat T leukemia cells or MDA-MB-435 breast cancer cells were exposed to 200 μg/mL LfcinB for the indicated times, after which cytotoxicity was determined by JAM assay. Representative of three independent experiments. *Columns* (**A** and **B**) or *points* (**C**), mean % cell death of quadruplicate determinations; *bars,* SD.

inhibitor that is known to cause the apoptotic death of leukemic cells (38). We further confirmed that LfcinB was triggering apoptosis in human cancer cells by staining Jurkat T leukemia cells with FITC-conjugated Annexin V and propidium iodide following LfcinB treatment. Annexin V binds specifically to phosphatidylserine headgroups that translocate to cell surface during the earliest stages of apoptosis (34). A time-dependent increase in the percentage of Annexin V positive cells was observed after 2 and 4 hours of exposure to LfcinB (Fig. 2B). After 4-hour exposure to LfcinB, >60% of cells stained positive for Annexin V and negative for propidium iodide. The lack of propidium iodide staining of LfcinB-treated Jurkat T leukemia cells confirmed that these cells were not dying by necrosis. In addition, light microscopy showed that, in comparison with untreated control cells, LfcinB-treated Jurkat T leukemia cells exhibited the typical apoptotic characteristics of cell shrinkage and membrane blebbing (Fig. 2C).

Localization of the Cytotoxic Activity of LfcinB to a Sequence of 10 Amino Acids

We next identified the amino acid sequence that was responsible for the apoptosis-inducing activity of LfcinB. We hypothesized that the cytotoxic activity of LfcinB might reside within the same sequence of 10 amino acid (FKCRRWQWRM) and/or 6 amino acid (RRWQWR) residues that have been reported previously to be responsible for the antimicrobial activity of LfcinB (39, 40). A comparison of the apoptosis-inducing activity of the 10-mer and 6-mer peptide fragments of LfcinB with that

of intact LfcinB revealed that the 10-mer peptide fragment was approximately equivalent to intact LfcinB in its ability to induce DNA fragmentation in Jurkat T leukemia cells at equimolar concentrations over 18 hours of culture (Fig. 3). In contrast, the 6-mer peptide fragment was unable to cause any substantial DNA fragmentation. Taken together, these data indicated that the apoptosis-inducing activity of LfcinB was localized to a sequence of 10 amino acid residues that also has antimicrobial activity (39).

Caspase-2, Caspase-3, and Caspase-9 Are Required for LfcinB-Induced Apoptosis

We next determined the role of individual caspases in LfcinB-induced apoptosis. Figure 4A shows that treatment of Jurkat T leukemia cells with selective inhibitors of caspase-2 (Z-VDVAD-FMK), caspase-9 (Z-LEHD-FMK), or caspase-3 (Z-DEVD-FMK) before exposure to LfcinB had a significant inhibitory effect on subsequent induction of apoptosis, as did treatment with the general caspase inhibitor Z-VAD-FMK (data not shown). In contrast, inhibition of caspase-8 activity with Z-IETD-FMK did not substantially impair LfcinB-induced apoptosis. Similar results were obtained when MDA-MB-435 breast carcinoma cells were treated with LfcinB in the presence of selective caspase inhibitors (Fig. 4B). These findings suggested that LfcinB killed leukemia and carcinoma cells by activating caspase-2, caspase-3, and caspase-9 but not caspase-8, which was consistent with induction of the intrinsic pathway of apoptosis.

Immunoblot detection of cleavage products of specific pro-caspases was used to confirm the activation of caspase-2, caspase-3, and caspase-9 by LfcinB. Figure 5A shows the presence of cleavage products of pro-caspase-2 (34- and 27-kDa protein bands), pro-caspase-3 (17-kDa protein band), and pro-caspase-9 (35- and 17-kDa protein bands) in Jurkat T leukemia cells that were exposed to 200 μg/mL LfcinB for 2 hours. In contrast, there was no apparent increase in cleavage products of pro-caspase-8 (41- and 18-kDa protein bands) following LfcinB treatment. A time course analysis of caspase activity in lysates of LfcinB-treated Jurkat T leukemia cells revealed that caspase-2 was activated within 2 hours of LfcinB treatment (Fig. 5B). Substantial activation of caspase-3 was also observed at the 2-hour time point.

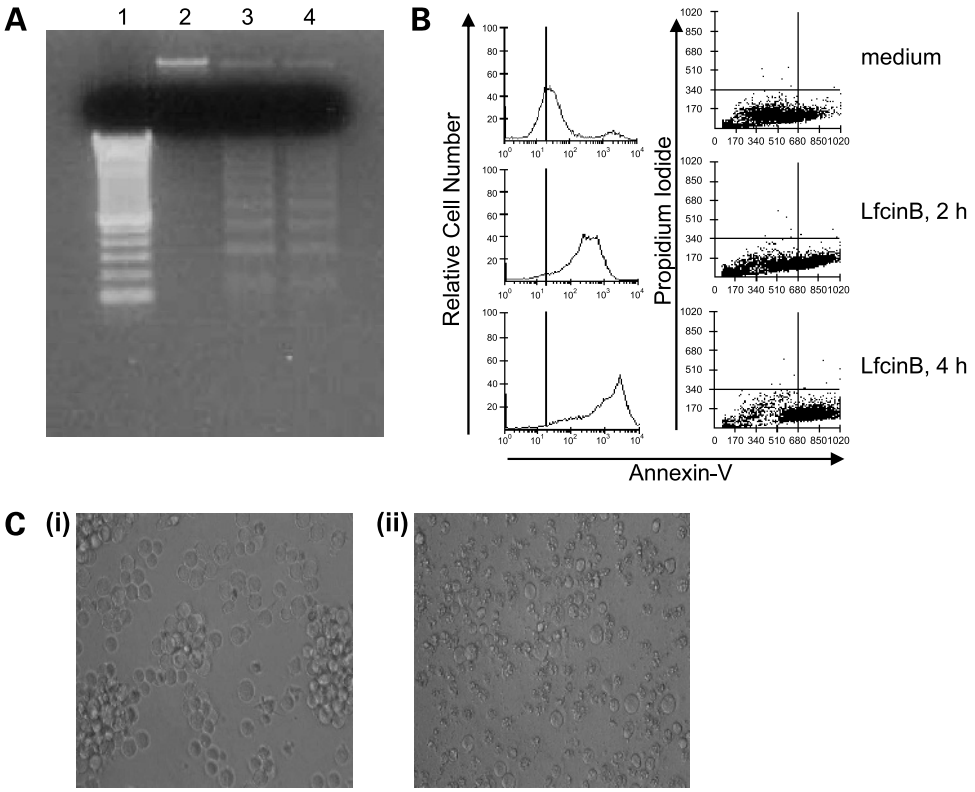

Figure 2. LfcinB induces apoptosis in Jurkat T leukemia cells. **A,** gel electrophoresis analysis of DNA fragmentation in Jurkat T leukemia cells following 4-h treatment with medium alone (*lane 2*) or 200 μg/mL LfcinB (*lane 3*) or 18-h treatment with 50 μmol/L etoposide (*lane 4*). *Lane 1* contained a standard 200-kb DNA ladder. **B,** flow cytometric analysis of Annexin V-FITC-stained Jurkat T leukemia cells before treatment and after 2- or 4-h treatment with 200 μg/mL LfcinB. *Bottom left quadrant,* living cells: 88.28% (untreated), 64.17% (2-h LfcinB), and 38.02% (4-h LfcinB); *bottom right quadrant,* Annexin V – positive cells: 11.57% (untreated), 35.65% (2-h LfcinB), and 61.67% (4-h LfcinB); *top left quadrant,* propidium iodide – positive cells: 0.13% (untreated), 0.10% (2-h LfcinB), and 0.17% (4-h LfcinB); *top right quadrant,* Annexin V – positive and propidium iodide – positive late apoptotic cells: 0.02% (untreated), 0.05% (2-h LfcinB), and 0.14% (4-h LfcinB). **C,** Jurkat T leukemia cells were cultured in the absence (*i*) or presence (*ii*) of 200 μg/mL LfcinB for 18 h and then photographed using a digital camera and a phase-contrast light microscope.

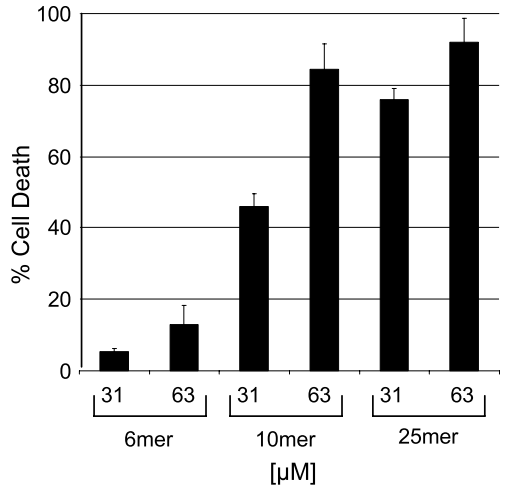

Figure 3. A 10 – amino acid derivative of LfcinB causes apoptosis. Jurkat T leukemia cells were cultured in the absence or presence of 31 or 63 μmol/L intact LfcinB (25-mer), 10 – amino acid (10-mer), or 6 – amino acid (6-mer) derivatives of LfcinB for 18 h. A 63 μmol/L concentration of the LfcinB 25-mer is equivalent to 200 μg/mL. Cytotoxicity was then determined by JAM assay. Representative of three independent experiments. *Columns,* mean % cell death of quadruplicate determinations; *bars,* SD.

After 4-hour exposure of LfcinB, caspase-2 and caspase-3 activity in Jurkat T leukemia cells had increased dramatically. Low-level caspase-8 and caspase-9 activation was detected at the 4-hour time point.

Failure to detect caspase-8 activity before caspase-3 activity as well as the failure of Z-IETD-FMK to prevent LfcinB-induced apoptosis argued against an important role for death receptors, although death receptors have been implicated in apoptosis induction by other anticancer agents (27, 41, 42). To confirm that LfcinB-induced apoptosis in cancer cells was independent of Fas, TRAIL DR4, and TRAIL DR5 death receptors, we used flow cytometry to measure Fas, Fas ligand, DR4, DR5, and TRAIL expression by Jurkat T leukemia cells following LfcinB treatment. There was no change in Fas, Fas ligand, DR4, or DR5 expression when cells were exposed to 200 μg/mL LfcinB, although a slight decrease in TRAIL expression was noted (data not shown). In addition, we treated Jurkat T leukemia cells with 200 μg/mL LfcinB in the absence or presence of neutralizing mAbs to Fas ligand or TRAIL at a concentration (10 μg/mL) that effectively inhibited Fas ligand– or TRAIL-induced apoptosis, respectively. Neither neutralizing mAb affected the ability of LfcinB to trigger apoptosis (data not shown), indicating that Fas/Fas ligand and/or TRAIL/DR4/DR5 interactions were not important for LfcinB-induced apoptosis.

LfcinB Causes Dissipation of the Mitochondrial Transmembrane Potential, Mitochondrial Swelling, and Cytochrome *c* Release

The involvement of caspase-2 and caspase-9 in the LfcinB-induced apoptotic death of Jurkat T leukemia cells suggested that LfcinB treatment might disrupt the membrane integrity of cancer cell mitochondria. We therefore employed the fluorescent dye $DiOC_6$, which localizes to intact mitochondria (33), to determine the effect of LfcinB on mitochondrial membrane integrity. Figure 6A shows a time-dependent leftward shift in $DiOC_6$ fluorescence of Jurkat T leukemia cells following exposure to LfcinB, indicating that LfcinB treatment resulted in the loss of mitochondrial membrane integrity and dissipation of mitochondrial transmembrane potential. In addition, Jurkat T leukemia cells that were engineered to overexpress the mitochondria-associated antiapoptotic protein Bcl-2 were rendered less sensitive to killing by LfcinB (Fig. 6B). The mitochondria of LfcinB-treated Jurkat T leukemia cells were also swollen in comparison with mitochondria of control cells (Fig. 7A,

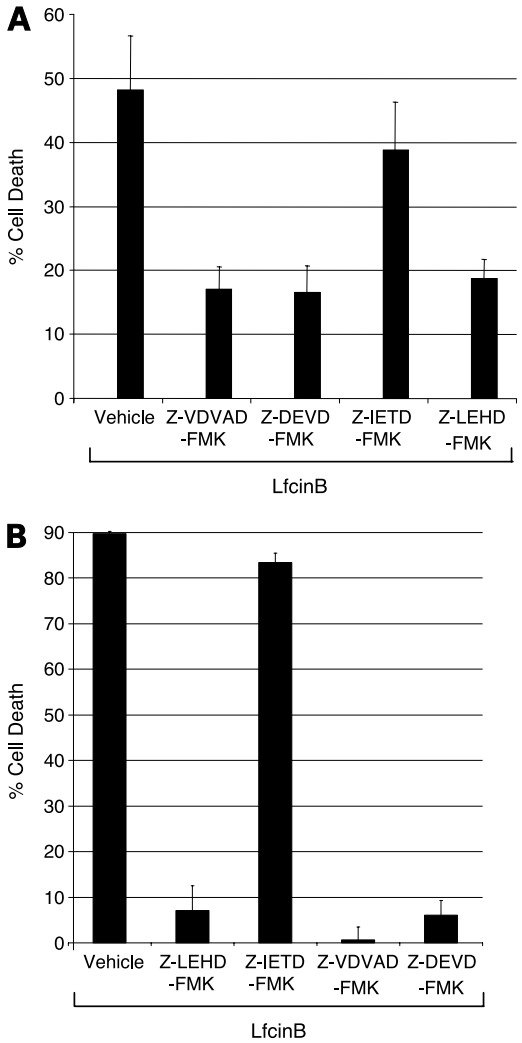

Figure 4. LfcinB-induced apoptosis is mediated by caspase-2, caspase-3, and caspase-9. Jurkat T leukemia cells (**A**) or MDA-MB-435 breast cancer cells (**B**) were treated with 200 μg/mL LfcinB in the absence or presence of an inhibitor of caspase-2 (Z-VDVAD-FMK), caspase-3 (Z-DEVD-FMK), caspase-8 (Z-IETD-FMK), or caspase-9 (Z-LEHD-FMK). After 18 h, cytotoxicity was determined by JAM assay. Representative of three independent experiments. *Columns,* mean % cell death of quadruplicate determinations; *bars,* SD.

ii versus *i*). Examination of 200 untreated and 200 LfcinB-treated Jurkat T leukemia cells revealed that >60% of mitochondria in LfcinB-treated cells were swollen (Fig. 7B). Some swollen mitochondria were also observed in untreated cells, which was most likely a reflection of background apoptosis that normally occurs in Jurkat T leukemia cell cultures. Importantly, Fig. 7C shows that LfcinB treatment resulted in the release of cytochrome *c* from mitochondria to the cytosol of Jurkat T leukemia cells. In contrast, no cytosolic cytochrome *c* was detected in control cells. Taken together, these data indicate that LfcinB induced the mitochondrial pathway of apoptosis in human cancer cells.

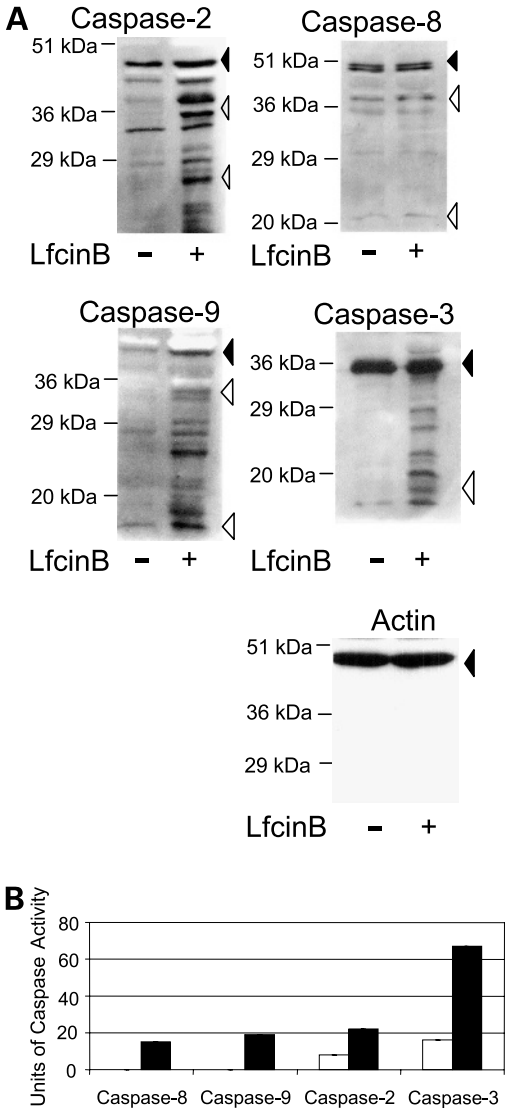

Figure 5. LfcinB treatment results in the cleavage of pro-caspase-2, pro-caspase-3, and pro-caspase-9. **A,** Jurkat T leukemia cells were treated with 200 μg/mL LfcinB for 2 h, washed, and lysed with CHAPS buffer. Cell lysates were clarified by centrifugation before immunoblot analysis using anti-caspase-2, anti-caspase-3, anti-caspase-8, and anti-caspase-9 antibodies. *Black arrowheads,* pro-caspase form; *white arrowheads,* major cleavage products. The blot was stripped between each antibody use. Actin expression was determined to confirm equal loading and transfer of protein samples. **B,** cell lysates were prepared from Jurkat T leukemia cells following 2-h (*white columns*) and 4-h (*black columns*) treatment with 200 μg/mL LfcinB and combined with chromogenic substrates that are selective for caspase-2, caspase-3, caspase-8, and caspase-9. Caspase activity was determined by absorbance at 405 nm. Representative of three independent experiments.

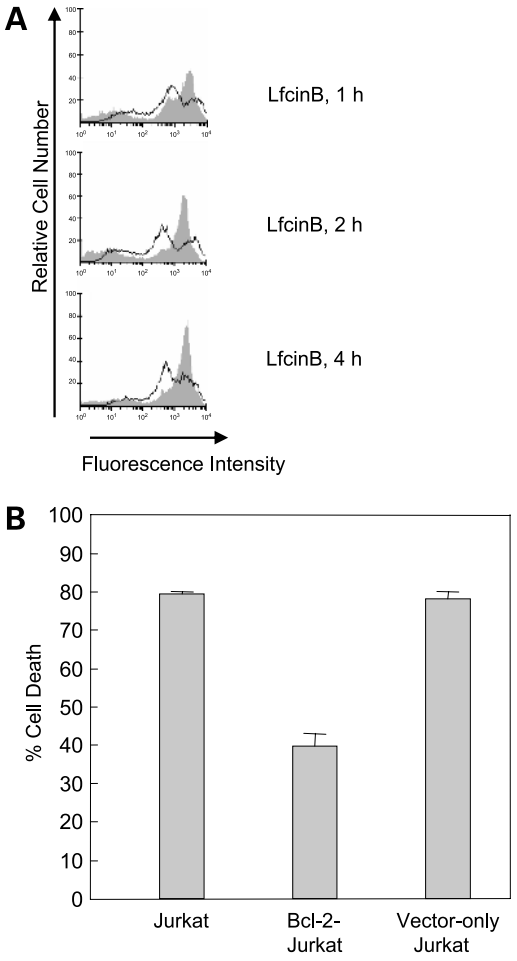

Figure 6. LfcinB-induced apoptosis is a mitochondria-dependent process. **A,** Jurkat T leukemia cells were cultured for the indicated times in the absence or presence or 200 μg/mL LfcinB. Cells were loaded with $DiOC_6$ (40 nmol/L) 30 min before the end of the assay. Loss of mitochondrial transmembrane potential is seen as a leftward shift in $DiOC_6$ fluorescence by LfcinB-treated cells (*open peak*) in comparison with untreated cells (*filled peak*) as determined by flow cytometry. **B,** unmodified Jurkat T leukemia cells, vector-only transfected cells, and Bcl-2-overexpressing cells were treated with 200 μg/mL LfcinB for 18 h. Cytotoxicity was determined by JAM assay. Representative of three independent experiments. *Columns,* mean % cell death of quadruplicate determinations; *bars,* SD.

Generation of ROS and Caspase-2 Activity Is Required for LfcinB-Induced Damage to Mitochondria

To determine whether exposure of cancer cells to LfcinB resulted in the generation of ROS, Jurkat T leukemia cells were loaded with dihydroethidium, which is oxidized to red fluorescent ethidium that accumulates in the nucleus

(35) and then treated with LfcinB. Flow cytometric analysis revealed a dramatic increase in fluorescence 1 hour after the cells were exposed to LfcinB, indicating the early generation of ROS (Fig. 8A). The antioxidant NAC ablated the LfcinB-induced accumulation of ROS in Jurkat T leukemia cells. Furthermore, LfcinB-induced cell death was reduced in a dose-dependent fashion when Jurkat T leukemia cells were exposed to LfcinB in the presence of increasing concentrations of the antioxidants glutathione or NAC (Fig. 8B).

We next showed that the LfcinB-induced generation of ROS in Jurkat T leukemia cells did not occur as a consequence of caspase activation because pretreatment with the broad-spectrum caspase inhibitor Z-VAD-FMK did not prevent intracellular accumulation of ROS in response to LfcinB treatment (Fig. 9A). However, activation of caspase-2 was a necessary condition for the LfcinB-induced reduction in mitochondrial membrane integrity because pretreatment of Jurkat T leukemia cells with the caspase-2 inhibitor Z-VDVAD-FMK prevented dissipation of mitochondrial transmembrane potential in response to LfcinB (Fig. 9B). In contrast, inhibition of caspase-3 or caspase-9 activity by pretreatment with Z-DEVD-FMK or Z-LEHD-FMK, respectively, did not prevent LfcinB-induced loss of mitochondrial transmembrane potential, implying that these particular caspases are activated downstream of caspase-2 as a consequence of diminished mitochondrial membrane integrity. Finally, the loss of mitochondrial transmembrane potential by Jurkat T leukemia cells in response to LfcinB treatment was partially reversed in the presence of the antioxidant NAC (Fig. 9C), suggesting that ROS are involved in the initiation of LfcinB-induced apoptosis.

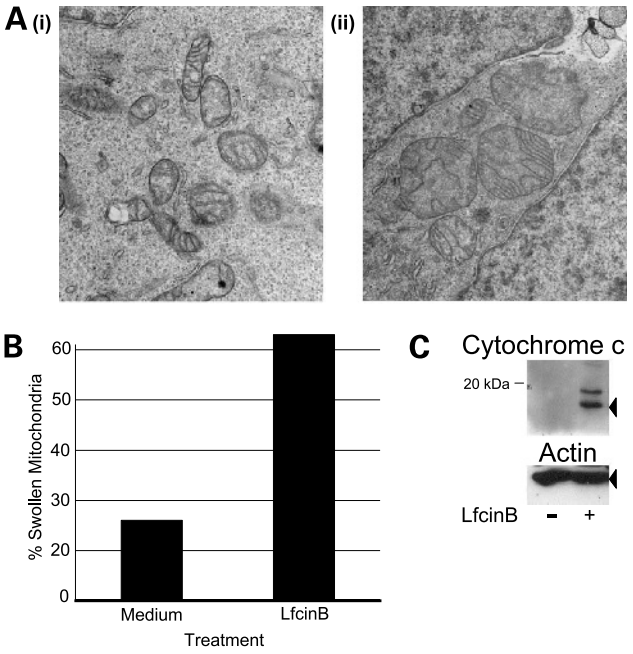

Figure 7. LfcinB treatment results in swelling of mitochondria and cytochrome *c* release. **A,** Jurkat T leukemia cells were cultured for 2 h in the absence (*i*) or presence (*ii*) of 200 μg/mL LfcinB, fixed with glutaraldehyde, and sectioned, and mitochondria were visualized by transmission electron microscopy. Magnification, ×30,000. **B,** % swollen mitochondria in untreated versus LfcinB-treated Jurkat T leukemia cells was determined by examining 200 untreated cells and 200 LfcinB-treated cells. **C,** Jurkat T leukemia cells were cultured in the absence or presence of 200 μg/mL LfcinB for 2 h, washed, and lysed with digitonin buffer. Cell lysates were clarified by centrifugation before immunoblot analysis using anti–cytochrome *c* antibody. The blot was then stripped and actin expression was determined to confirm equal loading and transfer of protein samples.

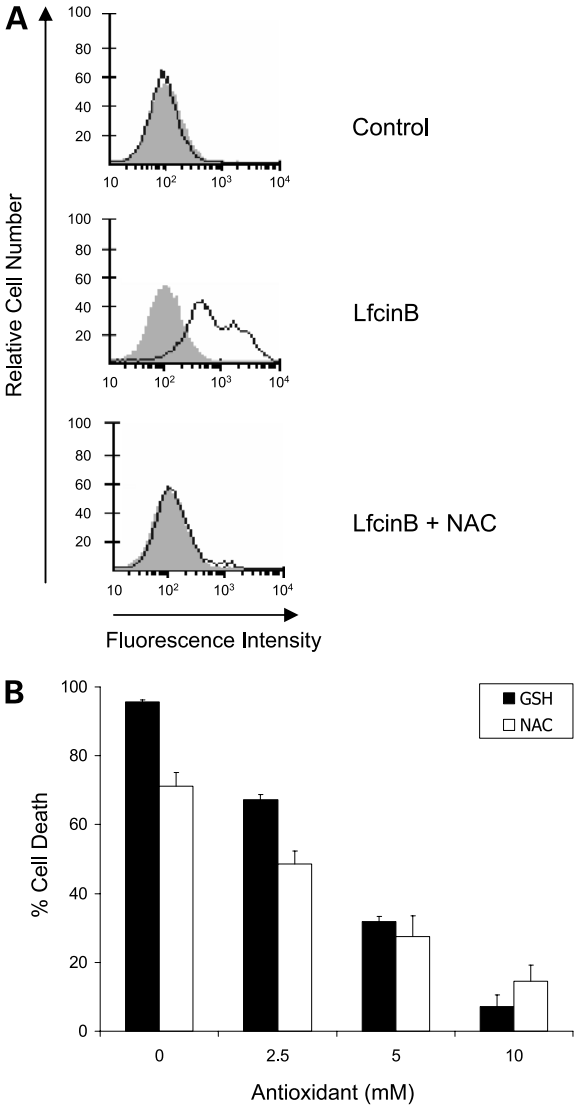

Figure 8. LfcinB-induced apoptosis involves the production of ROS. **A,** Jurkat T leukemia cells were cultured in the absence or presence of 200 μg/mL LfcinB without or with NAC for 1 h and then loaded with dihydroethidium. The presence of ROS was determined by flow cytometry (*open peak*) after 20 min; *filled peaks,* background fluorescence. **B,** Jurkat T leukemia cells were treated with 200 μg/mL LfcinB in the absence or presence of the indicated concentrations of glutathione (*GSH*) or NAC. After 18 h, cytotoxicity was determined by JAM assay. Representative of three independent experiments. *Columns,* mean % cell death of quadruplicate determinations; *bars,* SD.

Discussion

Chemotherapeutic drugs are widely used in cancer treatment but have the serious drawback of nonspecific toxicity because these agents target any rapidly dividing cell without discriminating between healthy and malignant cells (26). In addition, many neoplasms eventually become resistant to conventional chemotherapy due to selection for multidrug-resistant variants (43). The limitations associated with existing chemotherapeutic drugs have stimulated the search for new anticancer agents. In this study, we have shown that the milk-derived peptide, LfcinB, was cytotoxic for different human leukemia and carcinoma cells but did not have any adverse effect on the viability of normal human lymphocytes, fibroblasts, or endothelial cells. *In vitro* exposure to LfcinB caused DNA fragmentation in breast, colon, and ovarian carcinoma cell lines as well as in leukemia and lymphoma cell lines. In addition, DNA extracted from LfcinB-treated Jurkat T leukemia cells yielded a ladder-like pattern on agarose gel electrophoresis that is characteristic of apoptosis, whereas Annexin V staining detected the externalization of phosphatidylserine headgroups from the inner to the outer leaflet of the plasma membrane. LfcinB-treated Jurkat T leukemia cells also exhibited changes in cell morphology that included cell shrinkage and membrane blebbing. These findings indicated cell death by apoptosis (12) and agreed with an earlier report that LfcinB triggers apoptosis in cultures of THP-1 monocytic leukemia cells (11). Importantly, we have shown for the first time that LfcinB was able to cause apoptosis in carcinoma cell lines derived from malignancies of the breast, colon, and ovaries. Moreover, primary cultures of normal human lymphocytes, fibroblasts, or endothelial cells remained viable in the presence of LfcinB. Even human T cells that were rapidly proliferating in response to mitogenic stimulation were not adversely affected by a concentration of LfcinB that triggered apoptosis in cultures of Jurkat T leukemia cells.

Although the molecular basis of LfcinB selectivity for cancer cells is not yet clear, one possibility is that the cationic, amphipathic nature of LfcinB allows the peptide to interact with the negatively charged membrane of cancer cells but not with the neutral membrane of untransformed cells (44). In this regard, LfcinB has been shown to permeabilize and penetrate the bacterial cytoplasmic membrane (45), which also carries a net negative charge (44). Recently, LfcinB has been found to compromise the membrane integrity of murine fibrosarcoma cells (10), which may allow LfcinB to gain access to and disrupt the mitochondria of cancer cells. Some cancer cell lines used in our study (Skov3 and Caov3 ovarian carcinomas and T-47D breast carcinoma) were relatively resistant to LfcinB toxicity. One possible explanation is that more resistant cancer cell lines are less negatively charged and are therefore less able to complex with LfcinB via electrostatic interactions. Alternatively, more resistant cancer cell lines may overexpress antiapoptotic proteins, such as Bcl-x_L, as has already been shown for Skov3 cells (46). Nevertheless, the selective cytotoxic activity of LfcinB against different human cancer cell lines suggests a potential use for LfcinB in the treatment of certain human cancers but without the nonspecific toxicity associated with conventional chemotherapeutic agents. Ongoing studies seek to establish whether LfcinB is equally effective against cancer cells that express multidrug resistance genes.

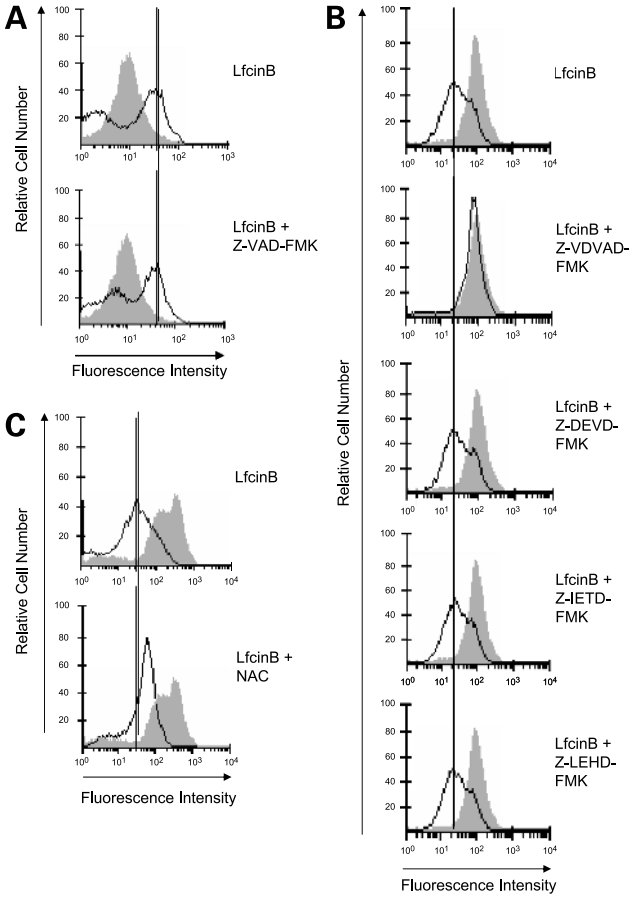

Figure 9. Sequential contribution of ROS and caspase-2 activation to LfcinB-induced changes in mitochondrial membrane integrity. **A,** Jurkat T leukemia cells were pretreated with the drug vehicle (DMSO) or 50 μmol/L Z-VAD-FMK and then cultured in the absence or presence of 200 μg/mL LfcinB plus the drug vehicle or Z-VAD-FMK for 1 h followed by loading with dihydroethidium. LfcinB-induced production of ROS was determined by flow cytometry (*open peak*) after 20 min; *filled peaks,* background fluorescence by cells treated with the drug vehicle or Z-VAD-FMK alone. **B,** Jurkat T leukemia cells were pretreated with the drug vehicle (DMSO) or 50 μmol/L Z-VDVAD-FMK (caspase-2 inhibitor), Z-DEVD-FMK (caspase-3 inhibitor), Z-IETD-FMK (caspase-8 inhibitor), or Z-LEHD-FMK (caspase-9 inhibitor) and then cultured for 2 h in the absence or presence of 200 μg/mL LfcinB plus the drug vehicle or the appropriate caspase inhibitor. Cells were loaded with $DiOC_6$ (40 nmol/L) 30 min before the end of the assay. Loss of mitochondrial transmembrane potential is seen as a leftward shift in $DiOC_6$ fluorescence by LfcinB-treated cells (*open peak*) in comparison with control cells (*filled peak*) as determined by flow cytometry. **C,** Jurkat T leukemia cells were cultured for 2 h in the absence or presence of 200 μg/mL LfcinB without or with 10 mmol/L NAC. Cells were loaded with $DiOC_6$ (40 nmol/L) 30 min before the end of the assay and flow cytometry was used to compare changes in mitochondrial transmembrane potential in LfcinB-treated cells (*open peak*) with control cells (*filled peak*). Representative of two independent experiments.

Native pepsin-generated LfcinB has a cyclic structure that is created by an internal disulfide bond that forms between cysteine residues at positions 3 and 20 (1). Our investigations revealed that a cyclic structure was not required for the anticancer activity of LfcinB because pepsin-generated LfcinB and synthetic LfcinB that lacked a cyclic structure were equally effective at inducing apoptosis in Jurkat T leukemia and MCF-7 breast carcinoma cell cultures. The antimicrobial activity of LfcinB is also equivalent for cyclic and linear forms of the peptide (39). In addition, equimolar concentrations of linear LfcinB and its 10–amino acid derivative (FKCRRWQWRM) were equally cytotoxic to Jurkat T leukemia cells, indicating that the apoptosis-inducing activity of LfcinB was localized to the NH_2-terminal region of LfcinB. Interestingly, the NH_2-terminal region of LfcinB contains three of five arginine (R) and one of three lysine (K) residues that confer a strong positive charge on LfcinB (44). However, the anticancer and antibacterial activities of LfcinB are not associated with precisely the same amino acid sequence because a 6–amino acid derivative (RRWQWR) of LfcinB that is reported to form the antimicrobial active center of the peptide (40) did not, in our hands, exhibit any cytotoxic activity against Jurkat T leukemia cells.

Inhibition of caspase-2, caspase-3, and caspase-9 activity protected Jurkat T leukemia cells and MDA-MB-435 breast carcinoma cells from LfcinB-induced apoptosis. In contrast, caspase-8 inhibition did not have any substantial effect on LfcinB-induced apoptosis. Immunoblot analysis using antibodies that were specific for activated caspases confirmed the presence of active caspase-2, caspase-3, and caspase-9 in Jurkat T leukemia cells 2 hours after exposure to a cytotoxic concentration of LfcinB. Little or no active caspase-8 was detected at this time, which argued against an important role for death receptors in LfcinB-induced apoptosis (16). Furthermore, flow cytometric analysis did not show any LfcinB-induced changes in Fas, Fas ligand, DR4, or DR5 expression by Jurkat T leukemia cells, although TRAIL expression was slightly decreased following LfcinB treatment. Moreover, the addition of neutralizing mAb to Fas ligand or TRAIL did not prevent LfcinB-induced apoptosis of Jurkat T leukemia cells. We conclude that exposure to LfcinB did not cause human cancer cells to die via the death receptor pathway of apoptosis, which is in contrast to the previously reported contribution of death receptor signaling to apoptosis caused by different chemotherapeutic drugs (41, 42).

Caspase-2 and caspase-9 function as initiator caspases for mitochondria-dependent apoptosis (18, 19). The involvement of caspase-2 and caspase-9, as well as the lack of death receptor involvement in LfcinB-induced apoptosis, implied an important role for the mitochondria-dependent pathway of apoptosis in LfcinB-mediated destruction of human cancer cells. Dissipation of mitochondrial transmembrane potential is commonly associated with destabilization of the outer mitochondrial membrane, which leads to the release of cytochrome *c* and other proapoptotic mitochondrial proteins into the cytoplasmic compartment (14). In fact, cell survival depends on the maintenance of mitochondrial transmembrane potential because of its involvement in ATP synthesis and maintenance of oxidative phosphorylation (47, 48). We observed that exposure to LfcinB resulted in a loss of mitochondrial transmembrane potential by Jurkat T leukemia cells. Furthermore, Jurkat T leukemia cells that were engineered to overexpress Bcl-2 were less susceptible to LfcinB-induced apoptosis. Bcl-2, which is localized on the mitochondrial membrane, inhibits the release of apoptogenic factors from mitochondria by several different mechanisms, including inhibition of proapoptotic Bax translocation from the cytoplasm to the mitochondria and trapping of activated proapoptotic ''BH3-only'' (e.g., Bid) Bcl-2 family members (49, 50). Proapoptotic Bcl-2 family members, such as Bax and Bid, function together with lipids to create membrane openings in mitochondria that allow the translocation of large mitochondrial proteins like cytochrome *c* (51). Collectively, our findings are consistent with LfcinB triggering activation of the intrinsic pathway of apoptosis in human cancer cells.

A kinetics analysis of caspase activity in the cytosolic fraction of LfcinB-treated Jurkat T leukemia cells suggested that caspase-2 activation preceded the activation of caspase-9. Inactive forms of caspase-2 and caspase-9 are present in both cytosolic and mitochondrial compartments, whereas only pro-caspase-2 is found in the nucleus (52, 53). We also determined that caspase-2 activity was a prerequisite for LfcinB-induced dissipation of mitochondrial transmembrane potential in Jurkat T leukemia cells. This latter finding was consistent with a recent report that nuclear pro-caspase-2 is cleaved to produce active caspase-2 in advance of cytochrome *c* release from mitochondria (54). In this regard, caspase-2 has been shown to control the cleavage of pro-caspase-9 by regulating mitochondrial membrane integrity, which in turn governs the release of cytochrome *c* involved in caspase-9 activation (55). We suspect that the low level of caspase-8 activity that was detected in LfcinB-treated Jurkat T leukemia cells following caspase-2 and caspase-3 activation was most likely due to a feedback amplification loop initiated by caspase-3 (56).

Caspase-3 is an important effector caspase (15, 21). Although active caspase-3 was present at high levels in LfcinB-treated Jurkat T leukemia cells, inhibition of caspase-3 activity did not completely prevent LfcinB-induced apoptosis. One possibility is that other executioner caspases, such as caspase-6 and caspase-7, also contribute to apoptosis caused by LfcinB. Alternatively, LfcinB may trigger the parallel involvement of apoptosis-inducing factor, which is released from mitochondria following the loss of mitochondrial transmembrane potential and mediates caspase-independent chromatin condensation and DNA fragmentation (57). Both scenarios are consistent with the finding that LfcinB triggered apoptosis in MCF-7 breast carcinoma cells, which lack pro-caspase-3 and also undergo cytochrome *c*–independent apoptosis (58, 59).

ROS, including the superoxide anion, hydroxyl radicals, and hydrogen peroxide, are normal byproducts of aerobic

metabolism (60). Each cell is therefore equipped with antioxidants, such as glutathione, that intercept ROS before they can cause harm to the cell. Apoptosis is frequently associated with oxidative stress caused by excessive depletion of glutathione and unchecked generation of ROS by the mitochondrial electron transport chain (61). Our data indicate that the production of ROS is an important early step that takes place before caspase activation during LfcinB-induced apoptosis in human cancer cells. Exposure to LfcinB caused increased production of ROS in Jurkat T leukemia cells, whereas the presence of exogenous antioxidants glutathione or NAC interfered with the apoptosis-inducing activity of LfcinB. In addition, the broad-spectrum caspase inhibitor Z-VAD-FMK did not prevent ROS generation in response to LfcinB. These findings are consistent with reports that implicate ROS in the induction of mitochondria-dependent apoptosis (25). Our data are also in good agreement with an earlier study that showed an important role for ROS in LfcinB-induced apoptosis of THP-1 leukemia cells (11). In addition to confirming these earlier findings, we have also shown that dissipation of mitochondrial transmembrane potential in LfcinB-treated Jurkat T leukemia cells is at least in part a consequence of oxidative stress caused by exposure to LfcinB. We speculate that initial ROS generation results from the permeabilization of negatively charged mitochondria by LfcinB that has penetrated the cancer cell membrane and entered the cytosolic compartment (10). This in turn leads to ROS-induced cleavage of pro-caspase-2 (62) followed by further destabilization of the outer mitochondrial membrane that amplifies ROS production and the caspase-2-regulated release of cytochrome *c*, which is required for the cleavage of pro-caspase-9 and downstream activation of caspase-3 (54). This sequence of events is consistent with the observation that NAC almost completely ablated ROS production and LfcinB-induced apoptosis in cultures of Jurkat T leukemia cells while only partially preventing dissipation of mitochondrial transmembrane potential.

Acknowledgments

We thank A. Bennett for technical assistance and M. Trevors and G. Faulkner for assistance with electron microscopy.

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Mol Cancer Ther 2005;4:612-624.