Sphingosine-Induced Apoptosis in Rhabdomyosarcoma Cell Lines Is Dependent on Pre-Mitochondrial Bax Activation and Post-Mitochondrial Caspases

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Abstract

Sphingolipids is the collective term ascribed to components of the sphingomyelin cycle. Modulation of the cellular levels of individual sphingolipids can induce a diverse range of cellular responses including apoptosis, proliferation, and cell cycle arrest. We present data showing that rhabdomyosarcoma cell lines, independent of lineage (alveolar rhabdomyosarcoma and embryonal rhabdomyosarcoma), are particularly sensitive to the induction of apoptosis as a result of an elevation in the cellular levels of sphingosine (d-erythro-sphingosine). Sphingosine-mediated apoptosis does not require its metabolism to the related proapoptotic molecule ceramide and is stereospecific because exposure of the rhabdomyosarcoma cell line RD to the 1-erythro and di-threo isomers of sphingosine did not induce apoptosis. Importantly, for efficient induction of apoptosis, sphingosine required Bax activation and consequential translocation to the mitochondria. This resulted in selective mitochondrial release of cytochrome c and Smac/Diablo but not other mitochondrial related factors (apoptosis-inducing factor, endonuclease G, and HtrA2/Omi). Using small interfering RNA, reduced Bax expression conferred the inhibiting factor, endonuclease G, and Smac/Diablo but not other mitochondrial related factors (apoptosis-inducing factor, endonuclease G, and HtrA2/Omi). Using small interfering RNA, reduced Bax expression conferred the impaired release of mitochondrial cytochrome c to the cytoplasm following sphingosine exposure, inhibiting the induction of apoptosis. Furthermore, dissipation of the inner mitochondrial membrane potential and enhanced production of reactive oxygen species were not observed. Bax activation and cytochrome c release were independent of caspases; however, caspase-3 and caspase-9 activity distal to the mitochondria was essential for the execution of apoptosis.

Introduction

Rhabdomyosarcoma describes malignant tumors of mesenchymal origin that arise from cells committed to a skeletal muscle lineage and is the most common soft-tissue sarcoma in children under the age of 15 years. Rhabdomyosarcoma tumors are characterized according to histology into two major categories, embryonal rhabdomyosarcoma and alveolar rhabdomyosarcoma, the latter being considerably more refractory to therapy (1). Novel therapeutic approaches that will permit progression in enhancing the survival of patients with rhabdomyosarcoma rely on identifying and understanding signal transduction pathways that result in, or prevent, rhabdomyosarcoma apoptosis. Apoptosis describes a series of evolutionarily conserved, tightly controlled mechanisms as a means to induce self-destruction, which is required to maintain normal tissue homeostasis. Cancer can therefore be partially attributed to a failure of cells to induce their apoptotic program (2).

Sphingolipids have a dual role within the cell, acting as key structural components of membranes as well as serving as bioactive second messengers. Central to the chemical backbone of all sphingolipids, sphingosine represents an intermediate linking the metabolism of ceramide to sphingosine-1-phosphate. Cellular levels of sphingosine are controlled by the action of ceramide synthase or sphingosine kinase, which are responsible for the formation of ceramide and sphingosine-1-phosphate, respectively. Conversely, sphingosine may be formed by the action of ceramidase from ceramide or sphingosine-1-phosphatase from sphingosine-1-phosphate (3, 4). Whereas an accumulation of ceramide and/or sphingosine functions as second messengers mediating cellular stress (5–9), accumulation of intracellular sphingosine-1-phosphate is related to survival. These opposing cellular responses mediated by intimately related molecules have led to the suggestion that a dynamic balance exists between ceramide, sphingosine, and sphingosine-1-phosphate, termed the “sphingolipid rheostat” (5–9).

Coupled with the effects of exogenous exposure of cells to ceramide(s) or the manipulation of enzymes responsible for ceramide accumulation, numerous studies have identified ceramide as a key inducer of cytotoxicity mediated by multiple agents that induce cellular stress including cytokines, chemotherapeutic agents, and ionizing radiation (4). More recent studies have identified a role for elevated levels of cellular sphingosine to mediate stress responses to the described classes of agents (5–7, 10), of relevance given their use in the first line treatment of rhabdomyosarcoma. However, unlike ceramide, the signal transduction pathways mediated by an accumulation of cellular sphingosine are ill defined.

Propagation and execution of apoptotic signals require the activation of the caspase cascade, a family of cysteine proteases. These have been subdivided into initiator (caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12) and executioner (caspase-3, -6, and -7) caspases. In addition to their crucial role as the energy-producing center of the cell, the mitochondria are central to the convergence of the intrinsic or extrinsic cellular signal transduction pathways that result in apoptosis. Essential to this process is the release of cytochrome c and other mitochondrial-related proteins that are normally confined to the mitochondrial intermembrane space by
antiapoptotic Bcl-2 family members (BH1–BH4 containing proteins; refs. 11, 12). Although mitochondrial expression of Bcl-2 and Bcl-X<sub>L</sub> can prevent cytochrome <em>c</em> release, BH1–3 only containing proteins, such as Bax and Bak, or BH3-only proteins Bad and Bid promote cytochrome <em>c</em> release on their activation and translocation to the mitochondria (13–15). Accordingly, cells lacking Bax, Bak, or both are resistant to apoptosis that requires the release of cytochrome <em>c</em> (16–19). However, the exact mechanism of cytochrome <em>c</em> release or other mitochondrial-related factors is controversial, and a number of hypotheses have been proposed (12, 20, 21). Some have suggested that Bcl-2 proteins may form channels in the outer mitochondrial membrane (22–24), form supramolecular channels (25), or alternatively interact with and regulate preexisting channels such as the permeability transition pore (12, 20, 26–28). Recent data suggest that oligomeric Bax is a component of the mitochondrial apoptosis–induced channel, and following their activation, a selective cytochrome <em>c</em> release pore is formed (29). However, the consensus of these theories is a requirement for Bax/Bak activation to promote pore opening and cytochrome <em>c</em> release whereas Bcl-2/Bcl-X<sub>L</sub> favors closure and maintenance of the mitochondrial cytochrome <em>c</em> pool.

Once released from the mitochondria, cytosolic cytochrome <em>c</em> complex with pro-caspase-9 and apa1-1 to form the apoptosome, triggering autoactivation of caspase-9, which subsequently acts as a vates caspase-3 and caspase-dependent nucleases. Alternatively, other proteins released from the mitochondria facilitate caspase activation by releasing their sequestration from the inhibitor of apoptosis proteins (11, 20). Also associated with cytochrome <em>c</em> release is the loss of mitochondrial membrane potential (ΔΨ<sub>m</sub>) and the production of reactive oxygen species (ROS; ref. 30). Indeed, the mitochondria are considered to be the primary source of ROS (21, 31). Further, the cellular redox state plays an essential role in regulating ceramide levels (32) and effecting cellular responses to elevation in ceramide (9), whereas elevated levels of mitochondrial-derived ROS are downstream effector molecules of cellular ceramide accumulation (8, 33).

The aim of this study was to further elucidate the apoptotic signal transduction pathway that is activated on an accumulation of cellular sphingosine in rhabdomyosarcoma. Direct evidence is provided for the requirement of Bax activation and its mitochondrial translocation in sphingosine-mediated apoptosis. Bax activation and the ensuing selective release of mitochondrial cytochrome <em>c</em> into the cytoplasm are independent of caspases. Furthermore, sphingosine-induced cytochrome <em>c</em> release and apoptosis occur independently of ROS production and loss of ΔΨ<sub>m</sub>. However, to induce a final commitment to apoptosis, caspase activation distal to mitochondrial cytochrome <em>c</em> release is required.

**Materials and Methods**

**Materials.** <em>v</em>-<em>erythro</em>-Sphingosine (sphingosine) and fumonisin <em>B</em><sub>1</sub> were obtained Biomol (Plymouth Meeting, PA). <em>v</em>-<em>erythro</em>-Sphingosine and <em>v</em>-<em>threo</em>-sphingosine were purchased from Matreya LLC Biochemicals (Pleasant Gap, PA). z-FA-fmk, z-VAD(OMe)-fmk, z-D(OMe)ED(OMe)-fmk, and z-LE(OH)HD(OMe)-fmk were purchased from MP Biomedicals (Aurora, OH). Unless stated, all other reagents were obtained from Sigma (St. Louis, MO). Sphingosine(s) were dissolved in anhydrous DMSO to a stock solution of 20 mmol/L and subsequent dilutions made with 2 mmol/L fatty acid–free bovine serum albumin (BSA). Vehicle treatments consisted of anhydrous DMSO dissolved in 2 mmol/L BSA to the same ratio as that containing sphingosine(s). Soluble tumor necrosis factor (TNF)–related apoptosis-inducing ligand was prepared as previously described (34).

**Cell culture and treatment.** The rhabdomyosarcoma cell lines Rh18, Rh36, and Rh41 were established at St. Jude Children’s Research Hospital (Memphis, TN). The rhabdomyosarcoma cell line RD, human embryonic kidney cell line HEK293, human foreskin fibroblasts, and murine skeletal myoblast cell line C2C12 were obtained from American Type Culture Collection. RD and Rh36 are of embryonal rhabdomyosarcoma origin and Rh18 and Rh41 of alveolar rhabdomyosarcoma origin. Cell lines were cultured in RPMI 1640 containing 10% characterized fetal bovine serum (Life Technologies/Invitrogen Corp., Carlsbad, CA) and 10 mmol/L l-glutamine. For apoptosis assays, cells were plated at a density of 250,000 per well in 12 well-plates; for protein extraction assays, 3 × 10<sup>5</sup> per 10-cm<sup>2</sup> Petri dish; and for immunoprecipitation assays, 1 × 10<sup>6</sup> in T75 culture vessels were harvested. After overnight attachment, cells were treated for up to 24 h with either vehicle alone, sphingosine, <em>v</em>-<em>erythro</em>-sphingosine, <em>v</em>-<em>threo</em>-sphingosine, or TNF-related apoptosis-inducing ligand. Where indicated, cells were pretreated for 60 min with fumonisin <em>B</em><sub>1</sub> (100–200 μg/mL), bongkrekic acid, cyclosporin A, z-FA-fmk, z-VAD(OMe)-fmk (z-VAD-fmk), z-D(OMe)ED(OMe)-fmk (z-D(OMe)ED-fmk), and z-LE(OH)HD(OMe)-fmk (z-LEHD-fmk; all 50 μmol/L) or for 4 h with catalase (1,000 u/mL or N-acetylcysteine (5 mmol/L).

**Retroviral expression vectors.** The retroviral expression vectors pMSCV-I-CrmA (expressing the viral caspase-8 inhibitor CrmA), pMSCV-I-DN-caspase-8 (expressing dominant negative mutant caspase-8), and pMSCV-I-DN-FADD (expressing the death domain only of Fas-associated death domain (FADD)), all also expressing green fluorescent protein (GFP) and separated by an internal ribosome entry site sequence, and the control vector pMSCV-I-GFP (expressing GFP) were kind gifts from Drs. Jill M. Lahti and Vincent J. Kidd (St. Jude Children’s Research Hospital) and have previously been described (34). Retroviral supernatants were prepared as previously described from HEK293T cells (34). RD cells were incubated overnight with viral supernatant in the presence of Polybrene (8 μg/mL; Sigma). After 24 h, the supernatant was discarded and RD cells incubated for a further 24 h with fresh viral supernatant. Viral transduced RD cells were then incubated at 37°C in nonviral media for an additional 48 h. The viral-transduced cells were sorted by expression of GFP using fluorescence-activated cell sorting (MoFlo, Dako, Ft. Collins, CO) and stable GFP-positive cells were selected. The expression of dominant negative FADD (indicated by the presence of a lower molecular weight band of the truncated protein), dominant negative caspase-8, and CrmA was confirmed by Western blot as described. The effect of transfection on RD cell growth was determined and plating density adjusted before treatment as for other rhabdomyosarcoma cell lines.

**Apoptosis assays.** Cells were detached in PBS/2 mmol/L EDTA, centrifuged at 1,000 rpm for 5 min, and gently resuspended in 250 μL of hypotonic fluorochrome solution (PBS, 50 μg propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100). The DNA content was analyzed by flow cytometry (Becton Dickinson FACS Calibur, San Diego, CA). Twenty-thousand events were analyzed per sample and apoptosis was determined from the sub-G<sub>1</sub>/G<sub>0</sub> DNA content (9).

**Western blotting.** After treatment, cells were detached in PBS/2 mmol/L EDTA, centrifuged at 1,000 rpm for 5 min, and lysed in 50 μL of ice-cold lysis buffer [10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 2 μg/mL aprotinin, 2 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L EDTA, and 1% Triton X-100]. Protein concentrations were determined with the Bio-Rad protein assay (Hercules, CA) according to the manufacturer’s instructions; 50 μg of protein were electrophoresed by SDS-PAGE (Bio-Rad). Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with anti–caspase-3 (31A1067; Alexis Biochemicals, San Diego, CA), anti–caspase–8 (5D3), anti–caspase–9 (clone 5B4), anti–Smac/Diablo (MBL, Watertown, MA), anti–poly(ADP-ribose) polymerase (C2-10), anti-Bcl-x<sub>L</sub> (clone 44), anti-Bcl-2 (clone 7), anti–FADD (clone 1), anti–CrmA (AT1-1), manganese superoxide dismutase (MnSOD; clone 19), cytochrome <em>c</em> (TH8.2C12; BD Transduction Laboratories, San Diego, CA), anti–endonuclease G (Axxora, San Diego, CA), anti–HtrA2/Omi
(229926; R&D Systems, Minneapolis, MN), anti-Bax (N-20; Santa Cruz Biotechnology, La Jolla, CA), anti-Bax (clone 6A7; Sigma), and β-actin (Abcam, Cambridge, United Kingdom) followed by horseradish peroxidase-conjugated antibodies (KPL, Inc., Gaithersburg, MD). The enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Inc., Rockford, IL) followed by exposure to CL-Xposure films (Kodak, Rochester, NY) was used to visualize proteins.

**Immunoprecipitation of active Bax:** To detect the active form of Bax, RD cells were collected and lysed in CHAPS lysis buffer as previously described (34). Briefly, activated Bax was immunoprecipitated from 350 μg of whole protein lysate per sample with the monoclonal antibody clone 6A7 (Sigma), which reacts only with Bax in its conformationally active state, and Protein G Sepharose 4 Fast Flow Beads (Amersham, Little Chalfont, United Kingdom). Isolated active Bax samples were then subjected to Western blot analysis and identified with a rabbit polyclonal anti-Bax (N-20; Santa Cruz) antibody and ECL as described above.

**Mitochondrial and cytosolic fractionation.** To determine the release of proapoptotic factors from the mitochondria to the cytosol by Western blot, PBS-washed RD cells were incubated on ice for 5 min in 100 μL of ice-cold CLAMI buffer (250 mmol/L sucrose, 70 mmol/L KCl, 100 μg/mL digitonin in PBS). The cells were pelleted (1,000 × g for 5 min at 4°C) and the supernatant containing cytosolic protein was stored at −80°C. The pellets were incubated at 4°C for 10 min in universal immunoprecipitation buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 0.2% Triton X-100, 0.3% NP40, 1× Complete protease inhibitor (Roche Diagnostics Corporation, Indianapolis, IN)]. The samples were centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant containing mitochondrial protein was stored at −80°C (35). For analysis of Bax activation in the mitochondrial and cytosolic fractions, the universal immunoprecipitation buffer used contained 0.5% CHAPS rather than Triton X-100/NP40. Proteins were subjected to Western blot analysis as described herein.

**Evaluation of mitochondrial membrane potential (ΔΨm) by flow cytometry.** RD cells in 12-well plates were loaded with JC-1 (5 μg/mL; Invitrogen) for 15 min before the end of the agent treatment period. Cells were harvested, washed twice with ice-cold PBS, and resuspended in PBS containing 0.25% BSA at a density of 1 × 10^6/mL. Samples were immediately analyzed for the ratio of JC-1 aggregates to JC-1 monomers by flow cytometry (BD FACS Calibur).

**Evaluation of cytosolic peroxide and superoxide levels.** Intracellular peroxide and superoxide levels were quantified by flow cytometry using the probes 2',7'-dichlorofluorescein diacetate and dihydroethidium (Invitrogen) as described elsewhere (9). Briefly, RD cells were loaded with 50 μmol/L 2',7'-dichlorofluorescein diacetate for 40 min or 10 μmol/L dihydroethidium

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**Figure 1.** Sphingosine mediates a rapid and dose-dependent induction of apoptosis in rhabdomyosarcoma cell lines. Rhabdomyosarcoma cell lines were exposed to d-erythro-sphingosine (sphingosine), l-erythro-sphingosine, or l-threo-sphingosine for 0 to 24 h and the percentage apoptosis determined from DNA cell cycle analysis of the sub-G0/G1 content (A–D). Alternatively, RD cells were pretreated with fumonisin B1 (FB1; 100/200 μmol/L) 1 h before sphingosine treatment for a further 24 h (D). RD or Rh41 cells were harvested after 6-h treatment, then lysed, and whole protein content was isolated before Western blot analysis for caspase-3, caspase-8, caspase-9, and poly(ADP-ribose) polymerase (PARP) processing with β-actin as a loading control (B and C). Points, mean of three to five independent experiments; bars, SE. Western blots are representative of three independent experiments.
for 15 min following exposure to sphingosine. Cells were then harvested, washed twice in PBS and 2',7'-dichlorofluorescein diacetate or dihydroethidium fluorescence, then analyzed by flow cytometry (BD FACSCalibur). A minimum of 10,000 cells were examined from each sample.

Small interfering RNA system and transfection. Small interfering RNAs (siRNA) were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The siRNA Bax consisted of a mixture of four siRNA duplexes targeting four different regions of the Bax mRNA (siGENOME SMARTpool Bax, M-003308-00). A pool of four nontargeting siRNA duplexes was used as a negative control (siCONTROL nontargeting siRNA pool, D-001206-13; siRNAcontrol). Transfection of cells with siRNA duplexes was done using Lipofectamine 2000 (Invitrogen). To determine the optimum conditions for Bax down-regulation, cells were transfected with 0, 25, 50, and 100 nmol/L of siRNAcontrol and siRNAmax for 0, 24, 48, or 72 h as recommended by Dharmacon. The down-regulation of Bax was determined by Western blot. Subsequently, cells were transfected with 100 nmol/L of siRNAcontrol or siRNAmax for 48 h and, following a change of media, treated for a further 24 h with the aforementioned agents.

Statistical analysis. Data are represented as the mean ± SE. In all cases, n refers to the number of independent experiments. Statistical analyses were done with the Student’s t test and ANOVA where P < 0.05 was considered significant.

Results

Exogenous exposure of rhabdomyosarcoma cell lines to the sphingolipid sphingosine induced apoptosis in a dose-dependent manner independent of rhabdomyosarcoma subtype (alveolar rhabdomyosarcoma versus embryonal rhabdomyosarcoma; Fig. 1A), which occurred with rapid kinetics (Fig. 1B and C) as quantified by flow cytometric analysis of the sub-G0/G1 content of DNA cell cycle histograms. This was consistent with the cleavage of

Figure 2. Sphingosine-mediated apoptosis essentially requires caspase-3 and caspase-9. RD cells were pretreated for 1 h with the pan-caspase inhibitor z-VAD(OMe)-fmk (PAN), inhibitor of caspase-3 z-D(OMe)E(OMe)-fmk (C3i), inhibitor of caspase-9 z-LE(OH)HD(OMe)-fmk (C9i), or the negative control z-FA-fmk (each at 50 μmol/L) before exposure to sphingosine (10 μmol/L) for a further 24 h (A and B) or 6 h (C). The percentage apoptosis was determined from DNA cell cycle analysis of the sub-G0/G1 content (A). Cell morphology was also evaluated by light microscopy (B). Caspase-3 activation or poly(ADP-ribose) polymerase processing was determined by Western blot analysis with β-actin as a loading control (C). Additionally, RD cells overexpressing GFP, CrmA, dominant negative caspase-8 (DNC8), or dominant negative FADD (DNFADD) were exposed to sphingosine (10 μmol/L) or TNF-related apoptosis-inducing ligand (50 ng/mL) for 24 h. Expressions of dominant negative FADD, dominant negative caspase-8, and CrmA are depicted by Western blot analysis with β-actin as a loading control (D). A and D, columns, mean of at least three independent experiments; bars, SE. Western blots and light microscopy images are representative of three independent experiments.
poly(ADP-ribose) polymerase, the processing of caspase-3, caspase-8, and caspase-9 (Fig. 1B and C), and the degradation of X-linked inhibitor of apoptosis (data not shown). Sphingosine-mediated apoptosis was specific to the naturally occurring form of L-erythro-sphingosine (here on referred to as sphingosine) because exposure of the rhabdomyosarcoma cell line RD to the stereoisomers L-erythro-sphingosine or L-threo-sphingosine did not result in significant apoptosis (Fig. 1D). In comparison with rhabdomyosarcoma cell lines, murine myoblasts (C2C12), HEK293 cells, and human foreskin fibroblasts showed resistance to the efficient induction of apoptosis mediated by sphingosine treatment (20 μmol/L; Supplementary Fig. S1). Furthermore, inhibition of ceramide synthase activity with the agent fumonisin B₁ did not inhibit sphingosine-mediated apoptosis, but rather showed a trend to increase sphingosine-mediated apoptosis, indicating that this cellular stress response was due to sphingosine accumulation and not its metabolism to the related molecule ceramide (Fig. 1D; refs. 5, 36).

Pretreatment with the broad-spectrum caspase inhibitor z-VAD-fmk, or those specific for caspase-3 and caspase-9 (z-DED-fmk and z-LEHD-fmk, respectively), abrogated sphingosine-mediated apoptosis (Fig. 2A–C). Overexpression of the viral inhibitor of caspase-8, CrmA, dominant negative caspase-8, or dominant negative FADD did not prevent sphingosine-induced apoptosis despite complete prevention of TNF-related apoptosis-inducing ligand–mediated apoptosis (Fig. 2D; ref. 34).

The mitochondrion acts as a central executioner in response to apoptotic stimuli, allowing signals from various inputs to converge (21, 31). Sphingosine induced the selective release of cytochrome c and Smac/Diablo from RD cells exposed to sphingosine (10 μmol/L). These proapoptotic mediators were observed to be initially released from the mitochondria 2 h posttreatment and were not accompanied by other factors including apoptosis-inducing factor, endonuclease G, or HtrA2/Omi (Fig. 3A). Release of cytochrome c from the mitochondria into the cytosol following sphingosine (10 μmol/L) treatment of RD cells was confirmed by confocal microscopy (data not shown). Inner mitochondrial membrane potential (ΔΨₘ), determined by the ratio of JC-1 aggregates to monomers, was not disrupted until after release of cytochrome c and Smac/Diablo and the appearance of apoptosis at 24 h posttreatment (Fig. 3B). Rottlerin (5 μmol/L) was used as a positive control for ΔΨₘ dissipation (37). Additionally, pretreatment of RD cells with bongkrekic acid or cyclopasin A, agents known to prevent permeability transition pore opening and cytochrome c release (20), did not disrupt sphingosine-mediated apoptosis (Fig. 3C). Sphingosine-induced apoptosis occurred without the elevated production of superoxide or peroxide measured using the probes dihydroethidium or 2,7'-dichlorofluorescein diacetate, respectively, and pretreatment

**Figure 3.** Sphingosine induces selective release of cytochrome c and Smac/Diablo without the loss of inner mitochondrial membrane potential (ΔΨₘ) or production of ROS. The rhabdomyosarcoma cell line RD was treated with sphingosine (10 μmol/L) for 0 to 4 h, and mitochondrial and cytosolic fractions isolated as described in Materials and Methods. A, mitochondrial and cytosolic fractions were examined by Western blot for MnSOD, apoptosis-inducing factor (AIF), HtrA2/Omi, endonuclease G (EndoG), Smac/Diablo, and cytochrome c (Cyto. C). B, loss of mitochondrial membrane potential (ΔΨₘ) was determined by flow cytometric evaluation of the ratio of JC-1 monomers (FL1) to JC-1 aggregates (FL2). ΔΨₘ was determined in RD cells loaded with JC-1 (5 μmol/L) for 30 min before the end of the sphingosine treatment period. Representative histograms obtained from flow cytometric evaluation of the ratio of JC-1 monomers (FL1) to JC-1 aggregates (FL2). RD cells were pretreated for 1 h with cyclopasin A (CsA; 10 μmol/L) or bongkrekic acid (BA; 50 μmol/L; C) or for 4 h with GSH (5 mmol/L) or catalase (CAT; 1,000 mU/mL; D) before the exposure of sphingosine for a further 24 h. The percentage apoptosis was determined from DNA cell cycle analysis of the sub-G₀/G₁ content. Intracellular superoxide (dihydroethidium (He)) and peroxide (2',7'-dichlorofluorescein (DCF)) were determined by flow cytometry as described in Materials and Methods (Q). Western blots and flow cytometry histograms are representative of three independent experiments.
of RD cells with the antioxidant N-acetylcysteine or catalase did not prevent sphingosine-mediated apoptosis (Fig. 3D). N-Acetylcysteine or catalase alone did not modulate the basal level of apoptosis in RD cells. Further, sphingosine-induced apoptosis was not inhibited by Bcl-2 or Bcl-xL overexpression (data not shown).

Cytochrome c release was associated with elevated levels of Bax within the mitochondrial fraction of RD cells treated with sphingosine (10 μmol/L). This was initially observed at 2 h accompanied by an associated loss of Bax from the cytoplasm (Fig. 4A). Total Bax levels were not modulated by sphingosine treatment (data not shown). Immunoprecipitation with an antibody that detects the activated form of Bax showed that Bax was activated after 2-h exposure of RD cells to sphingosine (Fig. 4B). Activated Bax was found primarily in the mitochondria following 4-h treatment with sphingosine (10 μmol/L; Fig. 4C).

Cytochrome c release and Bax activation were independent of caspases because pretreatment with the broad caspase-inhibitor z-VAD-fmk, or those specific to caspase-3 and caspase-9, did not prevent cytochrome c release (Fig. 5A) or Bax activation (Fig. 5B). Further, Bax activation occurred in the presence of CrmA, indicating that this was independent of caspase-8 (Fig. 5C).

Down-regulated expression of Bax in RD cells using siRNA Bax compared with wild-type or siRNA control-treated cells (Fig. 6B). This associates with the impaired release of cytochrome c observed in RD cells pretreated with siRNA Bax compared with siRNA control (Fig. 6C).

Discussion

The dysregulation of apoptosis is considered to be one of the six essential processes that underlie tumorigenesis. Defects in the activity of proapoptotic molecules are believed to be one mechanism by which cancer cells obtain a survival advantage and consequently acquire resistance to current chemotherapeutic strategies (2). The data presented show that sphingosine induces the selective release of cytochrome c and Smac/Diablo from the mitochondria independent of ΔΨm dissipation. Importantly, Bax translocation to the mitochondria and its activation are required for sphingosine-mediated cytochrome c release and the ensuing apoptosis. Although others have suggested the involvement of Bax in sphingosine-induced apoptosis in different model systems (36), this is the first direct evidence that Bax is required for efficient induction of sphingosine-mediated apoptosis. Given that Bax mutations have been described in humans diagnosed with colon or gastric cancer (38), our findings that sphingosine-mediated apoptosis requires Bax activation are of significant importance in delineating the loss of chemotherapeutic efficacy of agents that use shown). Induction of sphingosine-mediated apoptosis in RD cells was inhibited by siRNA Bax in RD cells when compared with wild-type or siRNA control-treated cells (Fig. 6B). This associates with the impaired release of cytochrome c observed in RD cells pretreated with siRNA Bax compared with siRNA control (Fig. 6C).
Bax, RD wt, RD-siRNAcontrol, or RD-siRNABax were treated with sphingosine fractions were isolated and the expressions of cytochrome c. DNA cell cycle analysis of the sub-G0/G1 content.

A (10 nmol/L) determined by Western blot analysis with MnSOD as a control for cytoplasmic control (siRNAcontrol). Expression of Bax following treatment with siRNAcontrol fumonisin B1 did not inhibit sphingosine-mediated apoptosis in to the related proapoptotic metabolite ceramide because to the accumulation of cellular sphingosine and not metabolism rhabdomyosarcoma cell lines evaluated. This was exclusively due rapid time- and dose-dependent induction of apoptosis in all sphingosine as a signaling intermediate to achieve their anti-molecular weight factors HtrA2/Omi, endonuclease G, or apopto-

Figure 6. Loss of Bax protects against sphingosine-mediated apoptosis. The rhabdomyosarcoma cell line RD was treated with siRNA directed against Bax (siRNAmax; 100 nmol/L) for 48 h. Scrambled siRNA duplexes were used as a control (siRNAcontrol). Expression of Bax following treatment with siRNAcontrol or siRNAmax was compared with levels in the untreated parental cell line by Western blot analysis with β-actin as a loading control (A). The percentage of knockdown was quantified by densitometry and normalized to β-actin levels. B, RD wt, RD-siRNAcontrol, or RD-siRNAmax were treated with sphingosine (10 μmol/L) for a further 24 h. The percentage apoptosis was determined from DNA cell cycle analysis of the sub-G0/G1 content. C, cytosolic and mitochondrial fractions were isolated and the expressions of cytochrome c and Bax were determined by Western blot analysis with MnSOD as a control for cytoplasmic contamination. A and B, columns, mean of three independent experiments; bars, SE. Western blots are representative of three independent experiments.

sphingosine as a signaling intermediate to achieve their anti-tumorigenic effects (6).

The administration of exogenous sphingosine resulted in a rapid time- and dose-dependent induction of apoptosis in all rhabdomyosarcoma cell lines evaluated. This was exclusively due to the accumulation of cellular sphingosine and not metabolism to the related proapoptotic metabolite ceramide because fumonisin B1 did not inhibit sphingosine-mediated apoptosis in RD cells. Similar findings have been described in Jurkat T cells (5) and other cell lines (36, 39), in contrast to apoptosis induced in MCF-7 cells in response to sphingosine that could be attributed to its conversion to ceramide (6). Collectively, these data indicate that the downstream signaling pathway stimulated in response to the cellular accumulation of sphingosine is cell type specific. Further, this induction of apoptosis is stereospecific because 1-threo-sphingosine and 1-erythro-sphingosine did not induce apoptosis (40).

The mitochondria act as a point of integration for apoptotic signals originating from both the extrinsic and intrinsic apoptotic pathways. We have shown that sphingosine induces the selective release of cytochrome c and Smac/Diablo in contrast to other proapoptotic factors, independent of caspases. However, activation of caspase-3 and caspase-9 distal to the mitochondria are essentially required to execute sphingosine-mediated apoptosis. We have used the tetrapeptide z-DEVD-fmk to inhibit caspase-3 activity. Although primarily used as an inhibitor of caspase-3, z-DEVD-fmk has also been shown to inhibit the activity of caspase-8 (41). However, because RD cells overexpressing dominant negative caspase-8, dominant negative FADD, or CrmA do not affect sphingosine-mediated apoptosis, the abrogation of sphingo-

sine-mediated apoptosis following z-DEVD-fmk pretreatment is a result of caspase-3 inhibition only. Although evidence for caspase-8 processing is apparent following treatment of RD cells with sphingosine as described here and elsewhere in Jurkat T cells (5), overexpression of CrmA or dominant negative caspase-8 did not inhibit the resulting apoptosis. These results are in contrast to that observed following the treatment of Jurkat T cells with phyto-

sphingosine, a structural analogue of sphingosine, where caspase-

8 processing is apparent early in the apoptotic signaling cascade by Bax. Alternatively, Bax could interact with voltage-dependent anion release channel induced early in the apoptotic signaling cascade by Bax. By this mechanism, Bax could interact with voltage-dependent anion channel, adenine nucleotide translocator, and cyclophilin D, components of the permeability transition pore (26–28). Here, permeability transition pore opening leads to loss of Δψm, fluid influx into the matrix leading to swelling and outer mitochondrial membrane rupture, and nonselective release of proapoptotic proteins (12, 20, 21). The data presented are supportive of the former as a mechanism for cytochrome c release in sphingo-

sine-mediated apoptosis because (a) selective release of cytochrome c, and to a similar extent Smac/Diablo, but not of the higher molecular weight factors HtrA2/Omi, endonuclease G, or apoptosis-inducing factor, was observed; (b) dissipation of Δψm was not observed until after the release of cytochrome c from the mitochondria and the appearance of fragmented DNA; and (c) mitochondrial ROS were not produced in excess following sphingosine treatment.

Several mechanisms have been described by which Bcl-2 family members may induce cytochrome c release (12, 20, 21). Bax activation and its insertion into the mitochondria results in its oligomerization and Bax pore formation. These selective cyto-

proteins (12, 20, 21). The data presented are supportive of the former as a mechanism for cytochrome c release in sphingosine-mediated apoptosis because (a) selective release of cytochrome c, and to a similar extent Smac/Diablo, but not of the higher molecular weight factors HtrA2/Omi, endonuclease G, or apoptosis-inducing factor, was observed; (b) dissipation of Δψm was not observed until after the release of cytochrome c from the mitochondria and the appearance of fragmented DNA; and (c) mitochondrial ROS were not produced in excess following sphingosine treatment.

The mitochondria act as a point of integration for apoptotic signals originating from both the extrinsic and intrinsic apoptotic pathways. We have shown that sphingosine induces the selective release of cytochrome c and Smac/Diablo in contrast to other proapoptotic factors, independent of caspases. However, activation of caspase-3 and caspase-9 distal to the mitochondria are essentially required to execute sphingosine-mediated apoptosis. We have used the tetrapeptide z-DEVD-fmk to inhibit caspase-3 activity. Although primarily used as an inhibitor of caspase-3, z-DEVD-fmk has also been shown to inhibit the activity of caspase-8 (41). However, because RD cells overexpressing dominant negative caspase-8, dominant negative FADD, or CrmA do not affect sphingosine-mediated apoptosis, the abrogation of sphingosine-mediated apoptosis following z-DEVD-fmk pretreatment is a result of caspase-3 inhibition only. Although evidence for caspase-8 processing is apparent following treatment of RD cells with sphingosine as described here and elsewhere in Jurkat T cells (5), overexpression of CrmA or dominant negative caspase-8 did not inhibit the resulting apoptosis. These results are in contrast to that observed following the treatment of Jurkat T cells with phyto-

sphingosine, a structural analogue of sphingosine, where caspase-

8 processing is apparent early in the apoptotic signaling cascade by Bax. Alternatively, Bax could interact with voltage-dependent anion release channel induced early in the apoptotic signaling cascade by Bax. By this mechanism, Bax could interact with voltage-dependent anion channel, adenine nucleotide translocator, and cyclophilin D, components of the permeability transition pore (26–28). Here, permeability transition pore opening leads to loss of Δψm, fluid influx into the matrix leading to swelling and outer mitochondrial membrane rupture, and nonselective release of proapoptotic proteins (12, 20, 21). The data presented are supportive of the former as a mechanism for cytochrome c release in sphingosine-mediated apoptosis because (a) selective release of cytochrome c, and to a similar extent Smac/Diablo, but not of the higher molecular weight factors HtrA2/Omi, endonuclease G, or apoptosis-inducing factor, was observed; (b) dissipation of Δψm was not observed until after the release of cytochrome c from the mitochondria and the appearance of fragmented DNA; and (c) mitochondrial ROS were not produced in excess following sphingosine treatment.

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Because dissipation of $\Delta W_m$ is not observed until after cytochrome $c$ release and the appearance of fragmented DNA, loss of $\Delta W_m$ seems to be as a consequence of sphingosine-mediated apoptosis and not a cause. This is compounded by the observations that pretreatment with bongkrekic acid or cyclosporin A, agents that promote closing of the permeability transition pore, did not inhibit sphingosine-induced apoptosis. These observations are in contrast to etoposide or C2-ceramide exposure of the T-cell hybridoma cell line 101, where bongkrekic acid prevented loss of $\Delta W_m$, cytochrome $c$ release, and apoptosis (43). Additionally, evidence for ROS production, a mediator and cause of $\Delta W_m$ dissipation (44, 45) in response to sphingosine, is lacking. Using a fluorescence-based approach, an elevated production of superoxide or peroxide levels within the cell could not be detected. Furthermore, reduced glutathione (GSH) and catalase did not modulate sphingosine-induced apoptosis in contrast to ceramide treatment (8, 9, 33).

The current data are also suggestive that not all cytochrome $c$ is released from the mitochondria and support the opinion that collapse of $\Delta W_m$ is not observed because mitochondrial cytochrome $c$ is maintained at a threshold that permits normal electron transport and, hence, $\Delta W_m$ maintenance. Alternatively, it is possible that cytochrome $c$ may reenter the mitochondria to maintain normal mitochondrial respiration (30, 35, 46). Liberation of cytochrome $c$ in response to sphingosine exposure was also independent of caspases. Taken with the observation that cytochrome $c$ release is not accelerated by caspase-8 activation (data not shown), these data argue against a necessary role for a mitochondrial feedback loop.

The kinetics of Smac/Diablo release from the mitochondria seems to be similar to that of cytochrome $c$ release as reported elsewhere following UV-light exposure (47, 48). However, this does not necessarily indicate that cytochrome $c$ and Smac/Diablo share the same mitochondrial release pore (20). Further, loss of X-linked inhibitor of apoptosis was observed following sphingosine treatment of rhabdomyosarcoma cells (data not shown), likely to be a result of the liberation of Smac/Diablo from the mitochondria. Cytosolic Smac/Diablo assists cytochrome $c$ in activating postmitochondrial caspases by sequestering X-linked inhibitors of apoptosis that inhibit caspase-3 or caspase-9 (11, 20).

The BH3 domain of Bax is hidden within the native protein but is exposed when Bax is activated (49). Bax activation occurred rapidly following sphingosine exposure of RD cells first observed after 2-h treatment. Like cytochrome $c$ release, Bax activation was not modulated by, and was independent of, caspases. We have not determined whether Bax activation following the cellular accumulation of sphingosine requires an intermediate molecule (e.g., truncated Bid) or is direct. It is possible that Bid may act directly on the mitochondria, independently of Bax. However, recombinant tBid has been shown to mediate the formation of nonspecific pores (50, 51) rather than the selective release of cytochrome $c$ and Smac/Diablo described in the current study following sphingosine treatment of RD cells. In support of these observations, Kashkar et al. (52) showed that UV light–induced ceramide accumulation stimulated Bax activation independent of caspase-8 and Bid. Collectively, the data described and those of others (50, 51) lend support to the concept of direct Bax activation by accumulation of proapoptotic lipid intermediates.

Loss of Bax expression in RD cells using siRNA targeted against Bax correlated with loss of apoptosis in response to sphingosine exposure. Similarly, it has recently been reported that apoptosis in response to C2-ceramide, C16-ceramide, or UV light–induced ceramide accumulation treatment essentially requires Bax (52, 53). However, absolute inhibition of sphingosine-induced apoptosis was not observed in RD cells and can partly be attributed to the incomplete depletion of Bax expression. Additionally, we have shown a failure to completely prevent sphingosine-mediated apoptosis as HCT116 Bax−/− cells (data not shown), suggesting the involvement of an additional factor.

In summary, we present evidence describing a requirement for Bax activation and translocation to the mitochondria in sphingosine-mediated apoptosis. This permits the selective release of cytochrome $c$ and Smac/Diablo without loss of $\Delta W_m$ and ROS production, indicating that mitochondrial function is not totally eliminated. Both activation of Bax and release of cytochrome $c$ are independent of caspase involvement; however, for subsequent execution of apoptosis in response to sphingosine, caspase-3 and caspase-9 processing is essentially required.

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Sphingosine-Induced Apoptosis in Rhabdomyosarcoma Cell Lines Is Dependent on Pre-Mitochondrial Bax Activation and Post-Mitochondrial Caspases
