Induction of Apoptosis and Potentiation of Ceramide-mediated Cytotoxicity by Sphingoid Bases in Human Myeloid Leukemia Cells*

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Prior studies demonstrated that ceramide promotes apoptotic cell death in the human myeloid leukemia cell lines HL-60 and U937 (J arvis, W. D., Kolesnick, R. N., Fornari, F. A., J r., Traylor, R. S., Gewirtz, D. A., and Grant, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 73-77), and that this lethal process is potently suppressed by diglyceride (J arvis, W. D., Fornari, F. A., J r., Browning, J. L., Gewirtz, D. A., Kolesnick, R. N., and Grant, S. (1994) J. Biol. Chem. 269, 31685-31692). The present findings document the intrinsic ability of sphingoid bases to induce apoptosis in HL-60 and U937 cells. Exposure to either sphingosine or sphinganine (0.001-10 μM) for 6 h promoted apoptotic degradation of genomic DNA as indicated by (a) electrophoretic resolution of 50-kilobase pair DNA loop fragments and 0.2-1.2-kilobase pair DNA fragment ladders on agarose gels, and (b) spectrofluorophotometric determination of the formation and release of double-stranded fragments and corresponding loss of integrity of bulk DNA. DNA damage correlated directly with reduced cloning efficiency and was associated with the appearance of apoptotic cytoarchitectural traits. At sublethal concentrations (750 nM), however, sphingoid bases synergistically augmented the apoptotic capacity of ceramide (10 μM), producing both a leftward shift in the ceramide concentration-response profile and a pronounced increase in the response to maximally effective levels of ceramide. Thus, sphingosine and sphinganine increased both the potency and efficacy of ceramide. The apoptotic capacity of bacterial sphingomyelinase (50 milliunits/ml) was similarly enhanced by either (a) acute co-exposure to highly selective pharmacological inhibitors of protein kinase C such as calphostin C and chelerythrine or (b) chronic pre-exposure to the non-tumor-promoting protein kinase C activator bryostatin 1, which completely down-modulated total assayable protein kinase C activity. These findings demonstrate that inhibition of protein kinase C by physiological or pharmacological agents potentiates the lethal actions of ceramide in human leukemia cells, providing further support for the emerging concept of a cytoprotective function of the protein kinase C isoenzyme family in the regulation of leukemic cell survival.

Recent investigation has examined the participation of sphingophospholipid- and glycerophospholipid-derived messengers in the regulation of leukemic cell survival. We (1, 2) and others (3) have demonstrated that increased intracellular availability of ceramide induces programmed cell death or apoptosis in the human myeloid leukemia cell lines HL-60 and U937. Ceramide interacts with at least two distinct intracellular target enzymes, ceramide-activated protein kinase (4–6) and ceramide-activated protein phosphatase (7–9). A cytotoxic role for ceramide-activated protein phosphatase and ceramide-activated protein kinase in ceramide action has been inferred, although the relative contributions of these enzymes to the initiation of apoptosis is presently uncertain (10, 11). A contrasting cytoprotective function of diglyceride and, therefore, of one or more isoforms of protein kinase C (PKC) is supported by several lines of evidence. Increased intracellular availability of diglyceride abrogates the initiation of apoptotic DNA damage by ceramide in both HL-60 and U937 cells (1, 2); this effect is mimicked by such diverse pharmacological PKC activators as the stage 1 tumor promoters phorbol dibutyrate (2) and phorbol myristate acetate (2, 3), the stage 2 tumor promoter mezerein (2), and the non-tumor-promoting macrocyclic lactone bryostatin 1 (2). Collectively, these findings have defined opposing cytotoxic and cytoprotective roles for ceramide and diglyceride and, by extension, for their respective target enzymes in the regulation of leukemic cell survival.

In further support of a central cytoprotective function for PKC, we have also described the induction of apoptosis in HL-60 cells by pharmacological agents that selectively inhibit activity of this isoenzyme family (e.g. calphostin C and chelerythrine) (12). The importance of sphingoid bases such as trans-4-sphingenine (sphingosine) and 4,5-dihydroxyphospholipid (sphinganine) as physiologically relevant inhibitors of PKC is well established (13). In addition, the cytotoxic properties of sphingoid bases and other, more complex, lysosphingolipids have been linked directly to inhibition of PKC (14). Both sphinganine and sphingosine have been shown to reduce proliferative capacity and long term viability in HL-60 cells (15). Ohta and co-workers recently examined the lethal actions of

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1The abbreviations used are: PKC, protein kinase C; PBS, phosphate-buffered saline; kbp, kilobase pair(s); SMase, sphingomyelinase.
sphingosine within the context of cellular maturation and proposed that endogenous sphingosine mediates apoptotic cell death following phorboid-induced terminal differentiation in HL-60 cells (16). Apart from those studies, however, little information is presently available concerning the apoptotic influences of sphingoid bases in human leukemia cells.

The present report describes biochemical characterizations of direct and indirect apoptotic properties of sphingoid bases in undifferentiated HL-60 cells. These findings demonstrate that acute exposure to sphingosine and other sphingoid bases potently elicits apoptosis as assessed by multiple criteria, including the induction of double-stranded DNA damage, loss of clonogenic potential, and appearance of apoptotic morphology. These results additionally reveal that co-exposure to either sphingoid bases or selective pharmacological PKC inhibitors at sublethal concentrations augments the apoptotic capacity of the lethal lipid messenger ceramide. This interaction is mechanistically consistent with our previous observations that, conversely, ceramide-mediated cell death is suppressed by diglyceride and pharmacological PKC activators (1, 2). Thus, it appears that the apoptotic response to ceramide is indirectly regulated by the combined actions of sphingosine and diglyceride, which respectively limit or extend the cytoprotective influence of PKC. Based upon these observations, we propose that the reciprocal influences of sphingoid bases and diglyceride on PKC coordinately modulate ceramide-mediated apoptosis in human myeloid leukemia cells.

EXPERIMENTAL PROCEDURES

Drugs and Reagents

Synthetic preparations of D-erythro-sphingosine and D-erythro-dihydroceramide were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). Other sphingosine derivatives (e.g., 3-keto-D-erythro-sphingosine, N,N-dimethylsphingosine), and synthetic short-chain preparations of ceramide (N-octanoylsphingosine) and dihydroceramide (N-octanoylsphinganine) were also obtained from Biomol. Synthetic diglyceride analogs (1,2-dioctanoyl-sn-glycerol, 2,3-dioctanoyl-sn-glycerol, and 1,3-dioctanoyl-rac-glycerol) were obtained from Sigma. All lipids were initially dissolved in 100% ethanol and stored at −70 °C. For experimental use, concentrated ethanol stocks of various sphingolipids were complexed at a 1:1 molar ratio with delipidated bovine serum albumin (fraction V; 2 mg in PBS) by vigorous mixing for 90 min at 37 °C; stable protein-bound sphingolipid preparations were stored at −20 °C. In contrast, glycerolipids were used directly as concentrated stocks in 100% ethanol. Bacterial plasmid preparations of sphingomyelinase (SMase; from Staphylococcus aureus) in a vehicle of 50% glycerol, 0.25 mM NaH2PO4, pH 7.5, were obtained from Sigma or from Biomol and stored at 4 °C. The selective PKC inhibitors calphostin C and chelerythrine (LC Services Corporation, Woburn, MA) were dissolved in sterile water, and stored at 4 °C. The mycoxin fumonisin B1 (Sigma) was dissolved in 100% ethanol immediately before use. Broynton 1 was obtained in lyophilized preparations from (a) Dr. George R. Pettit (Arizona State University, Tempe, AZ) or from (b) the Cancer Treatment Evaluation Program of the National Cancer Institute; bryosterol (0.5 mg/ml); low molecular weight DNA fragments were resolved electrophoretically in pulsed-field gels. The formation of rosette (300-kbp loop and fragments of these mixtures (corresponding to 2 × 106 cells)) were cast into precooled 85-μl block molds and allowed to solidify at 4 °C. The arsogare imbued lyses were then treated with 250 μg EDTA, 250 μg EDTA, 1% N-lauroylsarcosine, pH 8.0 containing provate K (200 μg/ml; Sigma) at 55 °C for 48 h. Deproteinated lystate plugs were rinsed in 250 μg EDTA, 250 μg EDTA, 1% N-lauroylsarcosine, pH 8.0, and recovered into 2.2% agarose gels (SeaKem Gold; FMC); high molecular weight DNA fragments were resolved by field-inversion electrophoresis at 6 V/cm for 24–28 h in 0.5 × Tris borate/EDTA buffer at 14 °C; pulse intervals were ramped from 0.5 × to 50.0 s, with an F/R ratio of 3.0. Gels were stained for 6 h in 0.5 × Tris borate/EDTA buffer containing ethidium bromide (0.5 μg/ml), and DNA fragments were visualized by UV transillumination. DNA molecular weight reference preparations (48.6-kbp ladder; Life Technologies, Inc.) routinely were run in parallel to facilitate estimation of the size of rosette and loop DNA fragments.

Preparation of Sphingosine Stereoisomers

Various sphingosine stereoisomers were synthesized and purified as described previously (17–19), D-erythro-sphingosine and L-erythro-sphingosine were prepared, respectively, from tert-butoxycarboxyloxycarbonyl-β-serine and tert-butoxycarboxyloxycarbonyl-L-serine of the Garner aldheyde (17) with lithium pentadecyl in hexamethylphosphoramide-tetrahydrofuran as described by Herold (18), followed by Birch reduction in lithium-ethanolamine as described by Garner (19). D- and L-three-isomers were prepared in a similar manner, with the exception that coupling reactions were performed with lithium pentadecyl in the presence of zinc bromide in diethyl ether (18). Stereoisomers were characterized as N-bi-phenyl-carboxamido derivatives of sphingosine by high performance liquid chromatography on the basis of elution from a chiral column in hexane/2-propanol (82:18).
Quantitative Analyses of DNA Damage

The formation and release of DNA fragments, and the corresponding breakage of bulk DNA were assessed as described previously (1, 2). To measure intracellular DNA fragments, pelleted cells (4 × 10^6 cells/pellet in quadruplicate) were resuspended in PBS and lysed by addition of 5 mM Tris-HCl, 30 mM EGTA, 30 mM EDTA, 0.1% Triton X-100 (fully reduced), pH 8.0 (yielding a final density of 10^6 cells/ml), with gentle mechanical agitation. The lysates were centrifuged at 45,000 × g at 4 °C for 40 min; to measure extracellular DNA fragments, aliquots of incubation medium were adjusted to 5 mM Tris-HCl, 30 mM EGTA, 30 mM EDTA, pH 8.0, and centrifuged at 20,000 × g at 4 °C for 40 min. The pellets were discarded, and the presence of non-sedimenting DNA fragments in the supernatant from lystate and medium extracts was determined by dilution in modified Tris-sodium/EGTA buffer (3 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, pH 8.0) containing 1.0 μg/ml bis-benzimide trihydrochloride (Hoechst 33258; Sigma), and monitoring net fluorescence in each sample (λex = 365, λem = 460). Final DNA values were calculated relative to highly purified calf thymus DNA calibration standard; values for all such responses are uniformly expressed as nanograms/micrograms DNA recovered or released from 10^6 cells, and reflect the absolute amount of non-sedimenting, low molecular weight DNA present in lystate and medium preparations. Corresponding loss of integrity of bulk DNA was determined by enhanced-fluorescence alkaline unwinding analysis as described previously (1, 2). Pelleted cells (8.25 × 10^6 cells/pellet in quadruplicate) were resuspended in cold PBS and subjected to timed alkaline denaturation in 0.1 M NaOH; denaturation was terminated by neutralization in 0.1 M HCl. Cells were then further diluted in PBS and lysed by addition of 200 mM K2HPO4, 50 mM EDTA, 0.16% N-laurylsarcosine with brief sonication. Damage to bulk DNA in cell lysates was quantified by spectrofluorophotometry in the presence of Hoechst 33258 (λex = 350, λem = 450); induction of strand breaks was demonstrated by reduction of net DNA fluorescence. Values were standardized against graded DNA strand-breakage induced by scaled irradiation from a [137Cs] point source (30–300 rads), and are expressed as rad-equivalents.

Clonogenic Assay

Because we have found that HL-60 cells resist uptake of trypan blue even in advanced stages of apoptosis, the use of dye exclusion was precluded in these studies as a valid index of diminished viability; even in advanced stages of apoptosis, the use of dye exclusion was precluded in these studies as a valid index of diminished viability; and potential. Pelleted cells were washed extensively and prepared for soft-agar cloning as described previously (1, 2). DNA values were determined by dilution in modified Tris-sodium/EGTA buffer (3 mM NaCl, 10 mM Tris-HCl, 1 mM EGTA, pH 8.0) containing 1.0 μg/ml bis-benzimide trihydrochloride (Hoechst 33258; Sigma), and monitoring net fluorescence in each sample (λex = 365, λem = 460). Final DNA values were calculated relative to highly purified calf thymus DNA calibration standard; values for all such responses are uniformly expressed as nanograms/micrograms DNA recovered or released from 10^6 cells, and reflect the absolute amount of non-sedimenting, low molecular weight DNA present in lystate and medium preparations. Corresponding loss of integrity of bulk DNA was determined by enhanced-fluorescence alkaline unwinding analysis as described previously (1, 2). Pelleted cells (8.25 × 10^6 cells/pellet in quadruplicate) were resuspended in cold PBS and subjected to timed alkaline denaturation in 0.1 M NaOH; denaturation was terminated by neutralization in 0.1 M HCl. Cells were then further diluted in PBS and lysed by addition of 200 mM K2HPO4, 50 mM EDTA, 0.16% N-laurylsarcosine with brief sonication. Damage to bulk DNA in cell lysates was quantified by spectrofluorophotometry in the presence of Hoechst 33258 (λex = 350, λem = 450); induction of strand breaks was demonstrated by reduction of net DNA fluorescence. Values were standardized against graded DNA strand-breakage induced by scaled irradiation from a [137Cs] point source (30–300 rads), and are expressed as rad-equivalents.

Cytology

Pelleted cells were resuspended in PBS, fixed in conventional cyto-centrifuge preparations, stained with 20% Wright-Giemsa stain, and reviewed by light microscopy. The occurrence and mode of cell death in each treatment group were determined based on morphological criteria outlined previously (1, 2, 12). At least 3 fields of 100 cells each were scored for each treatment by assessing the expression of cytoarchitectural characteristics of either apoptosis (cell shrinkage, condensation of nucleoplasm and cytoplasm, formation of membrane blebs and apoptotic bodies) or necrosis (cell swelling, nuclear expansion, deterioration of organelar membranes, gross cytolysis).

In Vitro Assay of Total Cellular Protein Kinase C Activity

Assay of total protein kinase C activity in crude cell homogenates was performed as described previously (58). Briefly, preparations of cell lysates were transferred to acetylated filter discs and added to reactions mixtures containing lysis buffer (20 mM Tris-HCl, 500 mM EDTA, 50 μM EGTA, pH 7.5), synthetic phospholipid, phorbol 12-myristate 13-acetate, and cytosolic synthetic substrate (acylated myelin basic protein N-terminal peptide AcMBP4–14). The reaction was initiated by the addition of 25 μCi of [γ-32P]ATP, 20 μM non-isotopic ATP, allowed to proceed for 5 min at 30 °C, and terminated by addition of cold ortho-phosphoric acid (1%, v/v). The filters were washed and radioactivity determined by conventional liquid scintillometry.

Western Analysis of cPKCa Expression

Cells were lysed in 2 × Laemmli buffer, sonicated briefly, and stored at –20 °C pending analysis. Cellular proteins (2 × 10^6 cell equivalents/condition) were resolved by electrophoresis on 12.5% polyacrylamide gels, and then transferred to nitrocellulose membranes. Membranes were sequentially incubated in (a) rabbit anti-human polyclonal antibody (1:5000; Santa Cruz) for 1 h and (b) goat anti-rabbit polyclonal antibody horseradish peroxidase conjugate (1:5000; Calbiochem) for 1 h; immunoreactive cPKCa was visualized by enhanced chemiluminescence.

RESULTS

Induction of Apoptosis by Sphingosine and Sphinganine—Apoptotic cell death in HL-60 cells is characterized by loss of proliferative capacity, double-stranded degradation of genomic DNA, and profound alterations of cellular morphology. Highly selective pharmacological PKC inhibitors induce apoptosis in HL-60 cells (12), raising the possibility that sphingoid bases mediate similar lethal influences in the physiological regulation of cell death. The capacity of sphingoid bases to promote apoptotic cell death therefore was examined in these cells. Exposure of HL-60 cells to synthetic preparations of sphingosine (So; 10 μM), sphinganine (Sa; 10 μM), or vehicle (Veh) for 6 h. Apoptotic DNA fragments were resolved on agarose gels as described under "Experimental Procedures." Panel A, resolution of loop (~50 kbp) DNA fragments by pulsed-field electrophoresis. Panel B, resolution of oligonucleosomal DNA fragments (~0.2–1.2 kbp) by static-field electrophoresis. Data shown are from a representative study performed four times with comparable results.

Figure 1. Induction of apoptotic DNA degradation by sphingoid bases. HL-60 cells were exposed to synthetic preparations of sphingo- sine (So; 10 μM), sphinganine (Sa; 10 μM), or vehicle (Veh) for 6 h. Apoptotic DNA fragments were resolved on agarose gels as described under "Experimental Procedures." Panel A, resolution of loop (~50 kbp) DNA fragments by pulsed-field electrophoresis. Panel B, resolution of oligonucleosomal DNA fragments (~0.2–1.2 kbp) by static-field electrophoresis. Data shown are from a representative study performed four times with comparable results.
Apoptotic Properties of Sphingoid Bases

The concentration-response characteristics of sphingoid base-induced apoptosis—The concentration-response characteristics of sphingosine action were determined in subsequent studies (Fig. 4). Exposure of HL-60 cells to sphingosine over a broad range of concentrations (0.001–100 μM) for 6 h decreased clonogenicity and increased expression of apoptotic cytoarchitecture in a concentration-dependent manner (Fig. 4A). These responses were inversely correlated (R² = 0.988). Both the loss of clonogenicity and the appearance of apoptotic morphology were evident at 1 μM and maximal at 10 μM, with respective EC₅₀ values.

The apoptotic responses of HL-60 cells to sphingosine and sphinganine were equivalent, consistent with similar efficacies reported for these lipids with respect to inhibition of PKC (14). Conversely, the corresponding N-acyl derivatives ceramide and dihydroceramide differed markedly in apoptotic capacity (Table I), in that ceramide potently induced DNA fragmentation, whereas dihydroceramide was ineffective. Thus, while the effects of sphingosine have been attributed to conversion to ceramide in some settings (23), the identical responses to sphingosine and sphinganine indicates that the lethal actions of sphingoid bases do not reflect artifactual accumulation of ceramide. This was confirmed in related studies involving the mycotoxin fumonisin B₁, which prevents N-acylation of sphingoid bases by inhibition of ceramide synthase (24).

Conversely, the corresponding enantiomer pair L-threo-sphingosine and D-threo-sphingosine revealed similar efficacies with respect to induction of apoptotic DNA damage. Both the accumulation of DNA fragments and breakage of bulk DNA in response to each isomer were equivalent, although the L-threo isomer frequently exhibited a slightly higher efficacy for this response (−15%). Structurally related sphingoid bases were also screened for potential apoptotic capacity in HL-60 cells (data not shown). For example, the methylated derivative N,N-dimethylsphingosine was somewhat more potent than sphingosine in the induction of apoptotic DNA damage (e.g. by 28%), whereas 3-ketosphingosine was essentially ineffective at promoting apoptosis.

Concentration-response Characteristics of Sphingoid Base-induced Apoptosis—The concentration-response characteristics of sphingosine action were determined in subsequent studies (Fig. 4). Exposure of HL-60 cells to sphingosine over a broad range of concentrations (0.001–100 μM) for 6 h decreased clonogenicity and increased expression of apoptotic cytoarchitecture in a concentration-dependent manner (Fig. 4A). These responses were inversely correlated (R² = 0.988). Both the loss of clonogenicity and the appearance of apoptotic morphology were evident at 1 μM and maximal at 10 μM, with respective EC₅₀ values.

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The apoptotic capacity of sphingosine did not exhibit stereospecificity (Table II), consistent with a specific involvement of PKC. Direct comparison of D-erythro-sphingosine with L-erythro-sphingosine and the corresponding enantiomer pair L-threo-sphingosine and D-threo-sphingosine revealed similar efficacies with respect to induction of apoptotic DNA damage. Both the accumulation of DNA fragments and breakage of bulk DNA in response to each isomer were equivalent, although the L-threo isomer frequently exhibited a slightly higher efficacy for this response (−15%). Structurally related sphingoid bases were also screened for potential apoptotic capacity in HL-60 cells (data not shown). For example, the methylated derivative N,N-dimethylsphingosine was somewhat more potent than sphingosine in the induction of apoptotic DNA damage (e.g. by 28%), whereas 3-ketosphingosine was essentially ineffective at promoting apoptosis.

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values of 2.1 and 2.4 \( \mu M \). The induction of apoptotic DNA degradation, as reflected by the formation and release of DNA fragments (Fig. 4B) and the corresponding breakage of bulk DNA (Fig. 4C), exhibited divergent concentration-response profiles. The concentration-response profile for sphingosine-induced DNA fragmentation was distinctly biphasic, reminiscent of the apoptotic responses to selective pharmacological PKC inhibitors reported previously (12). Significant (\( p < 0.01 \)) accumulation of DNA fragments was discernible at 1 \( \mu M \) and maximal at 10 \( \mu M \); above 10 \( \mu M \), the generation of DNA fragments declined progressively (to \(-40\% of the maximal fragmentation observed at 10 \( \mu M \)). As the total number of cells recovered at the end of the exposure interval was not appreciably diminished, the apparent reduction in the extent of DNA damage associated with exposure to sphingosine at higher levels (i.e. >10 \( \mu M \)) could not be attributed to release of DNA upon physical dissolution of dead cells. In contrast, the concentration-response profile for sphingosine-induced breakage of bulk DNA was linear, rather than biphasic. Significant (\( p < 0.01 \)) breakage of bulk DNA was detected at 0.1 \( \mu M \) and maximal at 25 \( \mu M \); above 25 \( \mu M \), however, DNA breakage appeared to remain constant. The disparate concentration-response profiles for sphingosine provided by these separate assays suggested a fundamental change in the nature of DNA damage at higher sphingosine levels, a supposition that was confirmed in electrophoretic analyses of apoptotic DNA fragments (Fig. 5). Concentration-related changes in the appearance of apoptotic DNA fragments were demonstrated on both pulsed-field gels (Fig. 5A) and static-field gels (Fig. 5B). DNA loop fragments were initially observed at 1 \( \mu M \) and persisted throughout the range of concentrations tested; these bands increased in intensity and assumed a more compact appearance as the sphingosine concentration was escalated to 10 and 100 \( \mu M \). In contrast, oligonucleosomal DNA fragment ladders were observed exclusively at 10 \( \mu M \), and were replaced by a very faint continuous streak of DNA at 100 \( \mu M \). Virtually identical electrophoretic profiles of apoptotic DNA fragments were obtained from HL-60 cells in response to sphinganine (not shown).

Potentiation of Ceramide-induced Apoptosis by Sphingoid Bases and Pharmacological Inhibitors of PKC—Previous investigations have demonstrated that ceramide mediates the induction of apoptotic cell death in HL-60 and U937 cells (1–3). In addition, we have shown that the apoptotic response to ceramide in these cells is attenuated or abolished by diglyceride and a variety of pharmacological PKC activators (1, 2). To test the converse possibility that ceramide-related apoptosis is potentiated by sphingoid bases, additional studies were conducted to assess the apoptotic capacity of ceramide in the absence or presence of sphingosine and sphinganine (Fig. 6). Exposure of HL-60 cells to ceramide at a maximally effective concentration (10 \( \mu M \)) for 6 h potently induced apoptotic DNA damage, increasing the net (i.e. intracellular and extracellular) accumulation of double-stranded DNA fragments to 1635 ± 245 ng/10^6 cells and bulk DNA breakage to 3315 ± 325 rad equivalents. Ceramide exposure also increased the fraction of cells expressing apoptotic traits to 36% (data not shown). Co-exposure to ceramide (10 \( \mu M \)) and either sphingosine or sphinganine at a sublethal concentration (750 nm) significantly (\( p < 0.001 \)) enhanced ceramide action, as reflected by both the net accumu-
lation of DNA fragments and the breakage of bulk DNA. In fact, the extent of ceramide-induced DNA damage was augmented by approximately 89–96% in the presence of either sphingoid base. Moreover, sphingosine and sphinganine increased the fraction of cells exhibiting apoptotic morphology, expressed as % control colony formation and % total cells. Panel B, spectrophotometric determination of the formation of DNA fragments, with calculated total accumulation of DNA fragments; values are expressed as micrograms of DNA/10^6 cells. Panel C, spectrophotometric determination of bulk DNA breakage; values are expressed as kilorad equivalents. All values reflect the mean ± S.E. of quadruplicate determinations. Data shown are from representative studies repeated four times with comparable results.

**FIG. 4.** Concentration-response characteristics of sphingosine action: quantitative studies. HL-60 cells were exposed to sphingosine (So) over a broad range of concentrations (0.001–100 μM) for 6 h. Multiple aspects of apoptosis were then quantified as before. Panel A, clonogenic capacity (●) and occurrence of apoptotic morphology (●), expressed as % control colony formation and % total cells. Panel B, spectrophotometric determination of the formation (●) and release (●) of DNA fragments, with calculated total accumulation of DNA fragments (●); values are expressed as micrograms of DNA/10^6 cells. Panel C, spectrophotometric determination of bulk DNA breakage (●); values are expressed as kilorad equivalents. All values reflect the mean ± S.E. of quadruplicate determinations. Data shown are from representative studies repeated four times with comparable results.

**FIG. 5.** Concentration-response characteristics of sphingosine action: qualitative studies. HL-60 cells were exposed to sphingosine (So) over a broad range of concentrations (0.001–100 μM) for 6 h. Apoptotic DNA fragments were then separated on agarose gels as before. Panel A, resolution of DNA loop fragments by pulsed-field electrophoresis. Panel B, resolution of oligonucleosomal DNA fragments by static-field electrophoresis. Data shown are from a representative study performed six times with comparable results.

In other studies, HL-60 cells were exposed to ceramide over a broad range of concentrations (0.0001–100 μM) in the absence or presence of sphingosine at a fixed (subeffective) concentration (750 nM) for 9 h (Fig. 7). As reported previously (1), ceramide produced a linear concentration-related increase in the accumulation of double-stranded DNA fragments. Sphingosine produced a distinct increase in the response to ceramide, consisting of both (a) a marked leftward shift in the ceramide
HL-60 cells were exposed to ceramide at a maximal concentration of ceramide (C8Cer; 5 μM) for 6 h in the absence or presence of various isomers of sphingosine (So; 10 μM) or diacylglycerol (10 μM) for 6 h. Apoptotic DNA damage was then assessed by quantitative spectrofluorophotometry as before. Panel A, formation (single-hatched bars) and release (double-hatched bars) of double-stranded DNA fragments; values are expressed as nanograms of DNA/10^6 cells. Panel B, loss of integrity of bulk DNA (solid bars); values are expressed as rad equivalents. Data shown are from a representative study performed four times with comparable results. All values reflect mean ± S.E. of triplicate determinations.
Sphingosine and sphingamine markedly limit proliferative capacity of HL-60 cells (13, 14), and their PKC-inhibitory effectors of the PKC isoenzyme family (13, 14), and C8Cer (Fig. 9, panel A) or bryostatin 1 (1,2-sn-diCer) at equimolar concentrations (10 μM). Apoptotic DNA degradation was quantified by spectrophotometry. Values reflect mean ± S.E. of triplicate determinations.

**Table IV**

| Treatment          | Intracellular DNA (μg/10^6 cells) | Extracellular DNA (μg/10^6 cells) | Bulk DNA breaks (rad eq) |
|--------------------|-----------------------------------|-----------------------------------|-------------------------|
| Vehicle            | 223 ± 22                          | 44 ± 7                            | 155 ± 18                |
| C8Cer              | 1671 ± 241                        | 559 ± 87a                         | 7270 ± 492a             |
| 1,2-sn-diCer + C8Cer | 253 ± 47b                        | 52 ± 12b                          | 194 ± 55b               |
| 2,3-sn-diCer + C8Cer | 1620 ± 139a                       | 505 ± 86a                         | 6720 ± 418a             |
| 1,3-rac-diCer + C8Cer | 1680 ± 343a                       | 549 ± 53a                         | 6948 ± 330a             |

a Increased vs. vehicle (p < 0.001).
b Decreased vs. C8Cer (p < 0.001).

**Fig. 9. Potentiation of sphingomyelinase-induced apoptosis by down-modulation of PKC.** HL-60 cells were treated with synthetic ceramide (N-octanoylsphingosine (C8Cer); 10 μM) for 9 h following pretreatment with either vehicle (veh) or bryostatin 1 (BRY, 250 nM) for 24 h. Total accumulation of apoptotic DNA fragments was then assessed by quantitative spectrophotometry as before; values are expressed as micrograms of DNA/10^6 cells. Data shown are from a representative study performed three times with comparable results. Values reflect mean ± S.E. of triplicate determinations.

**Fig. 8. Potentiation of sphingomyelinase-induced apoptosis by pharmacological inhibitors of PKC.** HL-60 cells were exposed to bacterial SMase (0.001–100milliunits/ml) in the absence (●) or presence (■) of either calphostin C (panel A) or chelerythrine (panel B) for 6 h. The total accumulation of apoptotic DNA fragments was then assessed by quantitative spectrophotometry as before; values are expressed as micrograms of DNA/10^6 cells. Data shown are from a representative study performed four times with comparable results. All values reflect mean ± S.E. of triplicate determinations.

(Sphingosine and sphingamine markedly limit proliferative capacity and viability (15), and recent evidence has suggested that this response involves the induction of apoptosis (16). Monocytoid differentiation in HL-60 cells is sustained by PKC activity (reviewed in Ref. 25), a well-defined process elicited by prolonged treatment with synthetic diglyceride (26), bacterial phospholipase C (27), or tumor-promoting phorboids (28–32). These responses are potently antagonized by sphingoid bases.

Induction of HL-60 cell differentiation by synthetic diglyceride is abolished by sphinganine (33). Phorbol-related maturation in these cells is similarly attenuated by both sphinganine (33, 34) and sphingosine (35), as well as by such diverse pharmacological inhibitors of PKC as isoquinoline derivatives (e.g. H7) (36), fungal metabolites (e.g. staurosporine), and acylcarnitines (e.g. palmitoylcarnitine) (37). Moreover, terminal monocytoid differentiation of HL-60 cells ultimately culminates in apoptotic cell death (38, 39). This process reportedly results from progressive, age-related increases in the intracellular availability of sphingosine, the apparent consequence of an augmented capacity to deacylate endogenous ceramide (16). Whether such alterations in sphingolipid metabolism represent an intrinsic feature of cellular maturation, or instead reflect a generalized feedback response to the sustained PKC activity necessary to support terminal differentiation, remains to be determined.

The present results demonstrate that sphingoid bases exert both direct and indirect apoptotic influences in myeloid leukemia cells. Acute exposure to sphingosine or sphinganine were found to (a) induce double-stranded degradation of genomic DNA, (b) suppress proliferative capacity, and (c) promote apoptotic cytoarchitectural changes. These findings are in agreement with qualitative characterizations of sphingosine-related apoptosis in HL-60 cells within the context of terminal differentiation described by Ohta and co-workers (16). The apoptotic actions of sphingosine and sphinganine exhibited essentially identical concentration-response profiles. A fundamental DISCUSSION

Sphingoid bases represent a versatile class of endogenous inhibitory effectors of the PKC isoenzyme family (13, 14), and thus have been found to suppress or attenuate numerous PKC-dependent aspects of leukemic cell survival. In HL-60 cells, sphingosine and sphinganine markedly limit proliferative capacity and viability (15), and recent evidence has suggested that this response involves the induction of apoptosis (16). Monocytoid differentiation in HL-60 cells is sustained by PKC activity (reviewed in Ref. 25), a well-defined process elicited by prolonged treatment with synthetic diglyceride (26), bacterial phospholipase C (27), or tumor-promoting phorboids (28–32). These responses are potently antagonized by sphingoid bases.

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change in DNA damage was noted at high sphingoid base concentrations (i.e. 10–25 μM), however. Specifically, whereas bulk chromatin was continuously cleaved into large (~50-kbp) DNA fragments, subsequent degradation of this high molecular weight material into small (~0.2–2.0-kbp) oligonucleosomal fragments was arrested. This phenomenon presumably reflects selective, concentration-related inhibition of the subtype(s) of apoptotic endonuclease responsible for internucleosomal hydrolysis of 50-kbp fragments. While such an underlying mechanism has yet to be demonstrated conclusively, this observation is consistent with reports suggesting that very early genomic lesions such as the initial breakage of static chromatin occur naturally in mammalian systems (49), the four isomers are equipotent in the inhibition PKC activity in vitro (35), and we observed a complete lack of stereoselectivity in the capacity of sphingosine to initiate apoptosis. Second, sphingosine and sphinganine are equipotent in the inhibition of PKC (suggesting that the trans-4 double bond is not essential for inhibition of PKC activity by sphingoid bases) (35), and we noted essentially identical apoptotic responses to sphingosine and sphinganine. An analogous relationship has been noted with respect to the physiological activation of PKC by diglycerides, in that 1,2-diradyl-sn-glycero 3-phosphate is stimulatory, whereas 1,3-rac-substituted species are inactive (50, 51). The application of such steric influences as criteria for implicating PKC in the mechanism of action of sphingoid bases and diradylglycerols also appears to be relevant in considering the modulation of ceramide action. Thus, the findings that the apoptotic capacity of ceramide was (a) comparably augmented by both D- and L- forms of sphingosine and sphinganine, and (b) selectively abolished by sn-1,2-substituted (but not sn-2,3-substituted or rac-1,3-substituted) forms of diglyceride additionally supports an involvement of PKC activity in the reciprocal modulation of ceramide action by sphingosine and diglycerides. Also consistent with an involvement of PKC in the apoptotic properties of sphingoid bases, down-modulation of PKC by chronic pre-exposure to brystatin 1 potentiated ceramide-induced apoptosis to essentially the same extent as did acute inhibition of PKC by sphingoid bases. In this regard, it is significant that the potentiated response to ceramide noted in PKC-down-modulated cells could not be further augmented in the presence of sphingosine or sphinganine.

While the biological actions of sphingosine have been attributed, under some circumstances, to N-acylation of sphingosine to form ceramide via the ceramide synthase pathway (23), the cytotoxic properties of sphingosine described in this report are unlikely to stem from such a process. First, as already noted, sphingosine and sphinganine exhibited equivalent potency and efficacy in both the direct induction of apoptosis and the potentiation of ceramide-dependent cell death. Conversion of sphingosine and sphinganine (i.e. dihydro sphingosine) to the corresponding N-acylated derivatives (i.e. ceramide and dihydroceramide, respectively) yields metabolites with disparate biological actions because the established bioeffector properties of ceramide, including the capacity to induce apoptosis, are not associated with dihydroceramide (3, 52). Second, and more significantly, both direct and indirect apoptotic influences of sphingosine were unaffected by the mycotoxin fumonisin B1. Because this toxin prevents the acylation of sphingosine to ceramide though competitive inhibition of ceramide synthase (Refs. 53 and 54; reviewed in Ref. 24), the actions of sphingosine described above more likely to reflect a direct action of sphingosine, rather than the artifactual accumulation of ceramide. Finally, it should be noted that, whereas a recent report describes transcriptional repression of multiple PKC isoforms in CV-1 monkey kidney cells following chronic treatment with fumonisin B1 (55), we found no evidence that acute (i.e. 6-h) exposure to 100 μM fumonisin B1 induced apoptosis in HL-60 cells.

The capacity of sphingoid bases to induce apoptosis is consistent with previous findings from this and other laboratories demonstrating that diverse exogenous inhibitors of PKC alone initiate this process (12, 56, 57). These results are also compatible with other studies indicating that the apoptotic efficacy of the potent antileukemic agent 1-[β-D-arabinofuranosyl]cytosine is augmented by manipulations that reduce cellular PKC activity, including both (a) down-modulation of PKC by...
chronic exposure to pharmacological PKC activators (58) and (b) inhibition of PKC by acute exposure to pharmacological PKC inhibitors (59). Furthermore, preliminary observations indicate that the ability of 1-β-arabinofuranosyl)cytosine to induce apoptosis in HL-60 cells is also subject to reciprocal modulation by diglyceride and sphingosine. Collectively, these findings have potentially important implications for targeting PKC in the development of novel antileukemic strategies. Indeed, the potential utility of sphingoid bases as antineoplastic agents has been noted by other investigators (60). Antitumor actions of sphingosine and structurally related compounds have been documented in numerous cell types (reviewed in Ref. 61). For example, sphingosine and other sphingoid bases profoundly reduce tumor cell number in vitro (62) and restrict tumor growth and metastasis in vivo (63). Similarly, synthetic structural analogs of sphingoid bases (e.g. stearylamine) have been found to inhibit the activity of PKC in purified preparations (33), and to exert potent antitumor influences both in vitro (64) and in vivo (65). Furthermore, recent observations by Schwartz and co-workers indicate that safingol (referred to elsewhere as SPC-100270), a synthetic preparation of l-threo-sphinganine, potently limits the extent of tumor cell invasiveness (66) and substantially augments the antineoplastic actions of such diverse agents as doxorubicin and mitomycin (67). Whether these interactions stem from potentiation of tumor cell apoptosis remains to be established.

In conclusion, these observations demonstrate that sphingoid bases promote apoptotic cell death in human myeloid leukemia cells through both direct and indirect mechanisms. Within the context of physiological regulation of apoptosis, the potentiation of ceramide-induced cell death by sphingoid bases directly complements our previous observations that diglyceride opposes ceramide action. On the basis of these findings, therefore, it is proposed that (a) the regulation of leukemic cell survival depends upon a balance between ceramide-driven systems (e.g. ceramide-activated protein kinase) and PKC, and that (b) the cytoprotective influence of PKC is modulated by the reciprocal actions of sphingoid bases and diradylglycerols.

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Induction of Apoptosis and Potentiation of Ceramide-mediated Cytotoxicity by Sphingoid Bases in Human Myeloid Leukemia Cells

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