In Vitro and in Vivo Induction of Apoptosis by Sphingosine and N,N-Dimethylsphingosine in Human Epidermoid Carcinoma KB-3-1 and Its Multidrug-resistant Cells

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ABSTRACT

Sphingolipid breakdown products, including ceramide and sphingosine, regulate cell growth, cell differentiation, and apoptosis. We examined the effect of various agents, including sphingolipids, on apoptosis induction in human epidermoid carcinoma KB-3-1 and its multidrug-resistant (MDR) subclone KB-C2 cells which express P-glycoprotein. Adriamycin (ADM) induced apoptosis in KB-3-1 cells but not in KB-C2 MDR cells at the concentration of 50 μg/ml. On the other hand, 15 μM sphingosine or its methylated derivative N,N-dimethylsphingosine (DMS) induced apoptosis in both cell lines in vitro. These results suggested that KB-C2 MDR cells were resistant to apoptosis induction by ADM but sensitive to that by sphingosine and DMS. Ceramide and sphingosine-1-phosphate, the initial metabolites of sphingosine, failed to induce apoptosis under the same experimental condition as sphingosine/DMS. The protein kinase C (PKC) inhibitors H7 and staurosporine did not induce apoptosis in either cell line, suggesting that PKC-independent signaling is involved in apoptosis induced by sphingosine and DMS, although both sphingosine and DMS have been shown to down-regulate PKC. Furthermore, DMS significantly inhibited the growth of KB-3-1 as well as KB-C2 MDR tumors in vivo, with evidence of increased apoptosis. The intracellular level of exogenously added [3H]sphingosine or [14C]DMS did not differ between the KB-3-1 parent cell line and its MDR subclone KB-C2, whereas that of [14C]ADM was reduced in KB-C2 MDR cells compared to KB-3-1 cells. These results suggest that P-glycoprotein acts as a transporter for ADM but not for sphingosine or DMS. Furthermore, DMS at the concentrations which induce apoptosis in KB-C2 cells did not affect the level of [14C]ADM. Because sphingosine and DMS induce apoptosis regardless of P-glycoprotein expression, they may provide a new strategy and a promising approach to the treatment of anticancer drug-resistant cancer.

INTRODUCTION

Sph³ has been shown to modulate transmembrane signaling through PKC-dependent or -independent pathways (1-4). We have demonstrated that treatment of human promyelocytic leukemia HL-60 cells with a phorbol ester increased the endogenous levels of ceramide and Sph and resulted in induction of differentiation and apoptosis (5, 6). Furthermore, Sph mimicked the phorbol ester in inducing apoptosis. Similarly, treatment of human neutrophils with tumor necrosis factor α caused apoptosis and increased endogenous Sph levels (7). These results suggest that endogenous Sph production may play an important role in regulating apoptosis in those cells. A metabolically stable, methylated derivative of Sph, DMS, was also shown to induce apoptosis in HL-60 cells and other solid carcinoma cells (8) and to inhibit tumor growth in vitro and in vivo (9).

It has been increasingly recognized that many anticancer agents act by inducing apoptosis (10, 12), and that insensitivity to apoptosis induction by certain agents may be due to multidrug resistance (10, 11). Overexpression of a membrane glycoprotein termed the P-glycoprotein is known to be one cause of multidrug resistance (13, 14) by its action as a pump molecule which transports hydrophobic anticancer agents out of the cells. P-glycoprotein is normally expressed in the epithelia of excretory organs and the endothelia of the blood-brain barrier, suggesting that it provides a protective mechanism against endogenous toxins produced metabolically and exogenous toxins present in the diet and environment (15). Sph has cationic amphiphilic properties which suggest it may serve as a substrate for P-glycoprotein (2). In this study, we examined whether Sph or...
DMS would induce apoptosis in MDR cells with high P-glycoprotein expression by using the epidermoid carcinoma cell line KB-3-1 and its MDR subclone KB-C2. We further examined the capability of the P-glycoprotein-expressing cells to pump these sphingolipids out of the cell using ADM for comparison.

MATERIALS AND METHODS

Materials. DMS (16) and Sph-1-phosphate (17) were synthesized as described previously. [14C]DMS was prepared from Sph with [14]CH₃¹ and K₂CO₃ reacted in a methanol solution. The following materials were purchased from the indicated suppliers: [³H]Sph (New England Nuclear, Boston, MA), [¹⁴C]doxorubicin hydrochloride (Amersham Corp., Arlington Heights, IL), bioitin-21-dUTP 3' end labeling kit (Clontech, Palo Alto, CA), avidin-horseradish peroxidase (ICN, Costa Mesa, CA), and Resolution TLC, autoradiography enhancer spray (L. M. Corp., Chestnut Hill, MA). All other reagents were purchased from Sigma Chemical Co.

Cell Culture. Human epidermoid carcinoma KB cells were obtained from Dr. M. M. Gottesman (NIH, Bethesda, MD). KB-3-1 cells were grown in DMEM containing 10% heat-inactivated fetal bovine serum supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin, 2 mM l-glutamine, and 1% sodium pyruvate. KB-C2 cells which express P-glycoprotein were originally selected from KB-3-1 cells with increasing concentrations of colchicine and maintained in 2 μg/ml colchicine (18, 19). KB-C2 cells were cultured without colchicine 2 or 3 days before the experiments.

Analysis of DNA Fragmentation. Cultured cells (4 × 10⁸) were treated with 15 μM C2-ceramide, DMS, Sph, or Sph-1-P, or with 50 μM H7 or 50 μg/ml ADM for 15 h at 37°C. All sphingolipids were stored in stock solution in 50% ethanol and diluted directly into culture media (final ethanol concentration < 0.1%). Control experiments were performed with ethanol (0.1%) as the vehicle. Cells were harvested, washed, and incubated in 10 mM Tris-HCl, 100 mM NaCl, 25 mM EDTA, and 0.5% SDS (pH 8.0) with 500 μg/ml proteinase K at 50°C until the mixture became clear. DNA was extracted as described previously (20) by phenol/chloroform and incubated with 0.3 mg/ml RNase A for 1 h at 37°C. After precipitation, DNA samples (~2 μg) were electrophoretically separated on a 1.5% agarose gel containing ethidium bromide. DNA was visualized by UV light and the gel was photographed with a Polaroid camera.

Measurement of Apoptosis by Flow Cytometry. Cultured cells (4 × 10⁶) were treated with different agents as described above. Apoptotic cells were quantitatively evaluated by flow cytometry as described previously (8). After 400 × g centrifugation, the resultant cell pellet was suspended in 0.3 ml hypotonic fluorochrome solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Samples were placed overnight in the dark at 4°C, and then the fluorescence of individual nuclei was analyzed using an EPICS flow cytometer (Coulter Electronics, Hialeah, FL).

Metabolism of [³H]Sph and [¹⁴C]DMS in KB Cells. KB-3-1 or KB-C2 cells were plated at 2 × 10⁶ cells in 0.5 ml media in 24-well plates and incubated at 37°C overnight. Cells were treated with 5 μM Sph including 9 nm [³H]Sph (0.1 μCi) or [¹⁴C]DMS (2 × 10⁴ cpn). At each indicated time, the media were removed and the reaction was terminated by the addition of 1.875 ml of ice-cold chloroform/methanol/concentrated HCl (100:200:1). Lipids were Extracted according to the method of Bligh and Dyer (21). Samples from the lower chloroform phase were dried, resuspended in small volumes of chloroform/methanol (2:1) solution, and applied to silica gel high-performance thin-layer chromatography plates (Merck, Darmstadt, Germany). The plates were developed in butanol/acetic acid/water (3:1:1), and bands were visualized by primulin and identified under UV light. After treatment of TLC plates with enhancer, autoradiography was performed with Kodak X-Omat film at ~80°C for 2 to 3 days. Radioactive areas were scraped and counted by a liquid scintillation counter.

Accumulation of [¹⁴C]ADM in KB Cells. Cells (3 × 10⁵/well in 24-well plates) were washed with Dulbecco’s PBS and incubated in 0.5 ml serum-free DMEM containing 50 μM HEPES with or without DMS for 30 min at 37°C. Cells were then incubated with 1.5 μM [¹⁴C]ADM (0.075 μCi) for 1 h at 37°C. After incubation, the cells were trypsinized and washed with PBS and the radioactivity was counted.

Cell Viability by MTT Assay. Cells (8 × 10⁶/well in 96-well plates) were cultured with various concentrations of DMS, ADM, or DMS plus ADM for 24 h at 37°C. Cell viability was assessed by a tetrazolium dye uptake assay (MTT) as described previously (22).

Liposome Preparation. Liposomes were prepared as described previously (23). Briefly, egg phosphatidylcholine/cholesterol/DMS (4.5:4.5:1, molar ratio) was dried under N₂ stream, hydrated in PBS, and sonicated by a bath-type sonicator (Laboratory Supply, Hicksville, NY) twice for 10 min with a 30-min interval.

Tumor Growth Inhibition in Vivo by Different Agents. The effect of DMS on tumor growth in vivo was examined and compared with that of other agents such as Sph-1-phosphate and ADM. Animals used for the present study were 8- to 9-week-old female BALB/c nude mice. KB-3-1 or KB-C2 cells (2-5 × 10⁶) were injected into the s.c. tissue in the backs of nude mice. Tumor volume was calculated with the formula (tumor length × width × height) × 0.5. When tumors reached the size of 100–200 mm³, treatment was initiated by i.p. injection of different agents. DMS and Sph-1-phosphate, each at 0.5 mg/mouse, were dissolved in 1.25% DMSO and ADM was in 0.9% NaCl. DMS or Sph-1-phosphate was administered three times at 8-15-h intervals on days 0 and 1. ADM at 0.17 mg/mouse (8 mg/kg) was administered on day 0.

Identification of Apoptotic Cells in Vivo using TUNEL Method. Tumor samples were removed 15 h after i.p. injection of 0.5 mg free DMS and fixed in 10% buffered formaldehyde. Apoptotic cells were stained for apoptosis using the TUNEL method as described previously (24). Tissue sections were incubated with 20 μg/ml proteinase K for 15 min at room temperature followed by blocking of endogenous peroxidase with H₂O₂. Sections were incubated with 0.6 units/ml terminal deoxynucleotidyltransferase and 0.75 μM biotin-dUTP in terminal deoxynucleotidyltransferase buffer (30 mM Tris-HCl (pH 7.2), 140 mM sodium cacodylate, and 1 mM CoCl₂) for 90 min at 37°C. The reaction was terminated with TB buffer (30 mM sodium citrate and 300 mM NaCl) and incubated with 4% BSA.
Fig. 1  Agarose gel electrophoresis of DNA from cells treated for 15 h with different agents. DNA was isolated from cells and analyzed by gel electrophoresis. The marker is HaeIII digest of dX 174DNA. A, KB-3-1 cells were treated with an ethanol vehicle (Control), 15 µM C2-ceramide (C2-cer), DMS, Sph, or Sph-1-phosphate (Sph-1-P). 50 µM H7, or 50 µg/ml ADM. B, KB-C2 cells were treated with an ethanol vehicle (Control), 15 µM DMS or Sph or 50 µg/ml ADM.

for 20 min. After incubation with horseradish peroxidase-avidin, sections were stained with 3,3’-diaminobenzidine buffer [50 mM Tris-HCl (pH 7.2), 50 µM/ml 3,3’-diaminobenzidine, and 0.007% H2O2] for 1 h at room temperature. Cells undergoing apoptosis were calculated as the percentage of positively stained nuclei per 10⁵ cells counted. The value was expressed as means ± SE from three independent experiments.

RESULTS

Analysis of DNA Fragmentation in KB Cells Treated with Sph or DMS.  Agarose gel electrophoresis showed that a 15-h exposure of KB-3-1 or KB-C2 cells to 15 µM Sph or DMS caused DNA fragmentation with a pattern characteristic of internucleosomal fragmentation (Fig. 1). In KB-C2 cells, DNA fragmentation was more apparent after treatment with DMS than with Sph. Ethanol vehicle (0.1%) as a control did not induce apoptosis. ADM at 50 µg/ml induced internucleosomal DNA fragmentation in KB-3-1 cells but not in KB-C2 cells. Other sphingolipid compounds, C2- or C6-ceramide, or Sph-1-phosphate, did not induce DNA fragmentation at similar concentrations in either cell line.

Measurement of Apoptosis Using Flow Cytometry.  The fragmented DNA content of apoptotic cells produced an unequivocal hypodiploid DNA peak that can be distinguished from the diploid peak (Fig. 2). Cells were treated with various agents for 4, 6, and 15 h. Treatment of cells with ethanol (Control) or C2-ceramide (C2-Cer, 15 µM) did not induce apoptosis during these time intervals. There was also no significant hypodiploid peak in cells treated with 15 µM Sph or DMS at the early stages (4 or 6 h, data not shown), whereas an apparent hypodiploid DNA peak was detected in both KB-3-1 and KB-C2 cells after a 15-h treatment (Fig. 2A). On the other hand, 50 µg/ml ADM caused apoptosis in KB-3-1 cells but not in KB-C2 cells. DMS in combination with ADM increased the apoptotic cells in KB-3-1 cells but not in KB-C2 cells as compared to DMS or ADM alone. Percentages of apoptotic cells treated with the various agents as measured by flow cytometry are summarized in Fig. 2B. Although Sph and DMS are known to be inhibitors of PKC activity, the PKC inhibitors H7 and
staurosorine did not induce DNA fragmentation (Figs. 1 and 2).

Metabolism of [3H]Sph and [14C]DMS in KB Cells. There was no significant difference in the uptake of [3H]Sph (Fig. 3, A and B) or [14C]DMS (Fig. 3, C and D) added exogenously to KB-3-1 or KB-C2 cells. [3H]Sph was readily incorporated into cells and reached 28% of the peak level after 1 min. The level of Sph gradually declined as Sph was converted into other sphingolipids; 24%, 17%, 11%, and 8% of added total radioactivity was present as Sph at 15 min, 1 h, 4 h, and 15 h, respectively. [3H]Sph acylated to ceramide was determined to be 5%, 14%, and 4% of the total added at 1, 4, and 15 h, respectively. Ceramide can be utilized in the synthesis of complex sphingolipids including sphingomyelin. Conversion of [3H]Sph into sphingomyelin was 0.5%, 7%, and 20% at 1, 4 and 15 h, respectively. On the other hand, conversion of [3H]Sph into Sph-1-phosphate (25) or glucosylceramide occurred later at 4 and 15 h, and the radioactivity was very low. In contrast to Sph, DMS incorporated into cells was not metabolized into any other sphingolipid and the total incorporation increased with longer incubation times; 27%, 35%, 45%, 70%, and 85% at 1 min, 15 min, 1 h, 4 h, and 15 h, respectively. Since the incorporations and subsequent metabolisms were apparently identical between KB-3-1 parent cells and its MDR cells, we concluded that the MDR phenotype induces no significant changes in the levels of either Sph or DMS.

Accumulation of [14C]ADM in KB Cells. Next, we examined the levels of incorporated ADM and the effects of DMS on its incorporation in both cell lines. As expected, the level of [14C]ADM incorporated by MDR KB-C2 cells after a 1-h incubation was nearly two times lower than that in the parent KB-3-1 cells. Furthermore, DMS did not affect the accumulation of [14C]ADM at the concentrations (5–15 μM) which apparently induce apoptosis in KB-C2 cells (Fig. 4). At a higher concentration (30 μM) DMS seemed to enhance the ADM accumulation. In parent KB-3-1 cells, DMS apparently enhanced the accumulation in a dose-dependent manner, although the mechanisms underlying the phenomenon are not clear at the present time. The radioactivity of [14C]ADM in KB-3-1 cells increased approximately 1.5 and 3 times by the preincubation with 15 and 30 μM DMS for 30 min as compared to the control (P < 0.05 and 0.01).
Fig. 5 Comparative effects of ADM, DMS, and ADM + DMS on growth of KB-3-1 and KB-C2 cells in vitro. KB-3-1 (B) or KB-C2 (A and C) cells were treated for 24 h with the indicated concentrations of ADM (A), DMS alone (B and C), or DMS with 5 μg/ml (B) or 100 μg/ml (C) ADM, and the cell viability was examined using the MTT assay. Each value represents the mean from three independent experiments. Bars, SE.

Identification of Apoptotic Cells in Vivo. Overall, KB-C2 tumors grew significantly slower than KB-3-1 tumors (Fig. 6). In control mice treated with 1.25% DMSO, the number of apoptotic cells in KB-C2 tumors was found to be higher than that in KB-3-1 tumors (KB-3-1, 1.4 ± 0.3; KB-C2, 4.0 ± 0.4; P < 0.5; Fig. 7, A and C). Furthermore, the number increased in both tumors when treated with free DMS at 0.5 mg/mouse (KB-3-1, 9.2 ± 0.6, P < 0.01; KB-C2, 12.3 ± 0.8, P < 0.01 versus control; Fig. 7, B and D).

DISCUSSION

Sph and DMS induced apoptosis in many epithelial carcinoma cells including human colon carcinoma HT29 and HRT18 cells and human epidermoid carcinoma A431 cells (8). However, much is unknown about the apoptotic downstream pathway of Sph and DMS. Activation of Sph-dependent protein kinases (30, 31) or dephosphorylation of the retinoblastoma (Rb) gene product (32) have been demonstrated, although any roles of these proteins in the apoptotic pathway remains unknown. We recently observed that Sph and DMS inhibited a mitogen-activated protein kinase in some epithelial carcinoma cells, resulting in the induction of apoptosis and/or bcl-2 down-regulation in HL-60 cells (33).

The incorporation and metabolism of Sph or DMS in vitro and/or in vivo did not differ significantly between the KB-3-1 parent and KB-C2 MDR cells. In contrast, the accumulation of [14C]ADM was reduced in the KB-C2 MDR cells as compared to the KB-3-1 parent cells. These results strongly suggest that P-glycoprotein acts as an active transporter for ADM, but not for Sph or DMS in vitro and/or in vivo. Consistent with this observation, DMS inhibited the in vivo growth of KB-C2 cells to the same extent as that of KB-3-1 cells, whereas ADM inhibited the growth of KB-3-1 cells but not that of KB-C2 cells. The extent of the inhibitory effects of DMS on in vivo tumor growth was apparently dependent on the injected formulation, so that the effect of the free DMS lasted less time than liposomal DMS, reflecting the short circulation life of the lipid in the blood (23). In this connection, the maintenance of high DMS concentration in the circulation will be crucial in a therapeutic application.

hand. ADM inhibited the growth of KB-3-1 tumors as expected, and these inhibitory effects apparently lasted longer than those of DMS in these cells, but ADM did not inhibit the growth of KB-C2 tumors at all (Fig. 6, B and C). The combination of DMS and ADM inhibited the growth of KB-3-1 tumors more than DMS or ADM alone. In contrast, their combination inhibited the growth of KB-C2 tumors to the same extent as DMS alone (Fig. 6C).

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Furthermore, KB cells used in our studies were more resistant to DMS as compared to other epithelial carcinoma cells, including human colon carcinoma HT29, human epidermoid carcinoma A431 cells (8), and MCF-7 cells, which showed apparent apoptosis in vitro with 5–15 μM DMS for 6 h. In vivo growth inhibition by DMS appears to be dependent on the susceptibility of cells to its apoptotic induction, as is often the case with anticancer agents.

Many lines of evidence have suggested a possible involvement of PKC in the phosphorylation of P-glycoprotein and increased drug resistance (34). Phosphorylation of P-glycoprotein has been reported to be significantly increased when resistant cells were treated with a phorbol ester, decreasing the drug accumulation and resulting in increased drug resistance (35). The PKC inhibitor staurosporine greatly inhibited the phosphorylation level of P-glycoprotein and, as a result, enhanced the accumulation of drugs in MDR cells including KB-C4 cells, a subline of KB-C2 cells (34, 36). These observations led us to explore the possibility that the function of P-glycoprotein might also be regulated by DMS, a known potent PKC inhibitor (16). DMS did not affect the accumulation of [14C]ADM at the concentrations (5–15 μM) which apparently induce apoptosis in KB-C2 cells (Fig. 4). However, at a higher concentration (30 μM), DMS seemed to enhance the ADM accumulation. On the contrary, recent work by Sachs et al. (37) has shown that the Sph derivative l-threo-dihydrosphingosine, safingol, modulated the MDR phenotype in MCF-7 DOX-resistant cell lines by inhibition of P-glycoprotein phosphorylation, correlating with its inhibitory action on PKC. l-threo-dihydrosphingosine increased [3H]vinblastine accumulation at 30 μM or at a higher concentration that does not induce apoptosis in these cell lines. The difference in apoptosis-inducing activities between DMS and l-threo-dihydrosphingosine might be significant since Schwartz et al. (27) reported that safingol alone at concentrations as high as 50 μM failed to induce apoptosis in mitomycin C-resistant gastric cancer cells, suggesting that the modes of action of these sphingolipids are different.

Most anticancer agents have been found to induce apoptosis regardless of their interaction with target molecules (10–12). Cellular metabolic damage or stress caused by anticancer agents may activate sphingomyelinase to initiate an increased ceramide concentration (28). The anthracycline daunorubicin has been recently shown to stimulate ceramide generation through activation of ceramide synthase but not sphingomyelinase, resulting in apoptosis in mouse P388 and human U937 leukemia cells (38). Many agents have been shown to activate sphingomyelinase to generate ceramide and cause apoptosis, although the role of ceramide generation in apoptosis induction was not determined. Because the activation of sphingomyelinase or the addition of exogenous sphingomyelinase is known to increase ceramide and Sph concentration, it is possible that Sph may be involved in the signaling pathway caused by anticancer agents as described above. Further study of lipid second messengers such as ceramide and Sph and their involvement in apoptotic pathways of other anticancer agents should prove to be enlightening.

Fig. 6  Effect of DMS or ADM on in vitro tumor growth of KB-3-1 and KB-C2 cells. When tumors reached a size of 100–200 mm3, treatment was initiated as described in “Materials and Methods.” A, KB-3-1 cells: mice were treated with DMSO (Control), Sph-1-phosphate (S-1-P), or free or liposomal DMS. B, KB-3-1 cells: mice were treated with ADM alone or with free DMS and ADM in combination. C, KB-C2 cells: mice were treated with DMS in free or liposomal form, ADM, or a combination of free form DMS and ADM. Points, mean tumor volume (V/V0); bars, SE. Statistical analysis as in Fig. 5. *, P < 0.05; **, P < 0.01. Each group consisted of five mice.

* E. A. Sweeney and Y. Igarashi, unpublished data.
Fig. 7 Identification of apoptotic cells in KB-3-I and KB-C2 tumors treated with DMS. Tumors were taken 15 h after i.p. injection of 0.5 mg/mouse free DMS. The percentage of apoptotic cells was calculated as described in "Materials and Methods." In control, apoptotic cells in KB-3-I tumors were rarely seen (A), whereas those in KB-C2 tumors (C) were frequently observed (KB-3-I. 1.4 ± 0.3; KB-C2. 4.0 ± 0.4; P < 0.05). Apoptotic cells increased in number in both KB-3-I tumors (B) and KB-C2 tumors (D) when treated with DMS (KB-3-I. 9.2 ± 0.6; P < 0.01; KB-C2. 12.3 ± 0.8; P < 0.01; versus control). Arrows, apoptotic nuclei that are positively stained. ×200.

Multidrug resistance caused by P-glycoprotein appears to be by-passed by Sph and/or DMS during the induction of apoptosis. However, it is well known that resistance to multiple drugs is conferred through many other mechanisms including multidrug resistance-associated protein (39, 40). Understanding which resistance mechanisms, if any, are involved with the apoptotic signaling pathway of Sph and DMS will further help the study of their therapeutic application in cancer.

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