

Synergistic Interaction between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer

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ABSTRACT

We have examined the mechanism by which sphingomyelin (SM) enhances chemotherapy in human pancreatic cancer cells, focusing on the correlation between ceramide metabolism and apoptosis. Dose response curves for gemcitabine in the absence or presence of 0.2 mg/mL SM provided IC_{50} values of 78.3 ± 13.7 and 13.0 ± 3.0 nmol/L, respectively. The cytotoxic effect of the combined treatment was synergistic (combination index = 0.36). Using annexin-V staining, the percentage of apoptotic cells was $3.6 \pm 2.6\%$ for the untreated cells, $6.5 \pm 3.8\%$ for the 0.2 mg/mL SM-treated cells, and $19.9 \pm 12.9\%$ for the 100 nmol/L gemcitabine-treated cells, but increased significantly to $42.1 \pm 12.7\%$ with the combined treatment ($P < 0.001$, compared with gemcitabine-treated group). The percentage of cells losing mitochondrial membrane potential followed a similar trend. The ceramide content of untreated and gemcitabine-treated cells was not significantly different (0.46 ± 0.29 and 0.59 ± 0.34 pmol ceramide/nmol PO_4). However, when 0.2 mg/mL SM was added, ceramide levels were 1.09 ± 0.42 and 1.58 ± 0.55 pmol ceramide/nmol PO_4 , for the SM alone and SM with gemcitabine-treated cells, respectively ($P = 0.038$). Acidic SMase was activated by exposure to gemcitabine but not SM, whereas the activities of neutral SMase and glycosylceramide synthase did not change with either gemcitabine or SM. The data are consistent with gemcitabine-induced activation of acidic SMase and indicate that the addition of SM can yield increased production of ceramide, mitochondrial depolarization, apoptosis, and cell death. Because SM by itself is relatively nontoxic, addition of this lipid to agents that induce apoptosis may prove useful to enhance apoptosis and increase cytotoxicity in cancer cells.

INTRODUCTION

Apoptosis is now recognized as a major pathway by which cytotoxic agents induce death of tumor cells. Attenuation of this response confers a drug-resistant phenotype that is frequently evident in malignancy (reviewed in ref. 1). Therefore, agents that facilitate apoptosis should improve therapeutic efficacy. Ceramide, a precursor to several bioactive lipids, and itself an intracellular signaling molecule, has a central role in both apoptotic and mitogenic pathways (2–8). Administration of water soluble ceramides to cells *in vitro* can initiate apoptosis. On the other hand, reducing intracellular ceramide levels through increased synthesis of glycosylceramide (9) or inhibition of sphingomyelinase (SMase) activity leads to drug resistance (10).

Activation of SMase is the predominant pathway for the generation of ceramide (3, 11, 12). Sphingomyelin (SM) is hydrolyzed by SMase to produce ceramide and phosphocholine. However, there is disagreement about which of several SMases is responsible for ceramide production. It has been reported that acidic SMase found in the caveolae, sphingolipid rich microdomains of the plasma membrane

that are involved in receptor-mediated signaling, is activated by exposure to drug (13). Other reports implicate neutral SMase found at the plasma membrane, as well as in mitochondria (2, 14–17).

We have hypothesized that at least one mechanism by which cancer cells avoid apoptosis is by a reduction in the availability of intracellular SM substrate, and that by administration of exogenous SM, it is possible to prime the response of the cells to apoptosis-inducing drugs. Implicit in this model is that normal cells will not be further sensitized by exogenous SM because they already maintain sufficient levels of intracellular SM. Thus, within cancer cells, the limiting factor to the initiation of apoptosis is the availability of SM, whereas in normal cells it is the activation of SMase(s). This notion is supported by our previous studies which found that SM, when given as a single agent to athymic (nude) mice bearing human colonic tumor xenografts, had no effect on tumor growth but was able to potentiate the antitumor effect of chemotherapy with 5-fluorouracil (5-FU; refs. 18 and 19). The enhanced efficacy of 5-FU was associated with increased levels of apoptosis, compared with tumor-bearing mice that received 5-FU without SM (20). SM enhanced the *in vivo* antitumor effect in all colonic tumor models examined and with multiple drugs. Importantly, inclusion of SM in the chemotherapy regimen did not increase toxicity. These results suggested that SM may be an ideal adjuvant to boost chemotherapeutic efficacy.

Although pancreatic cancer is only the eighth and ninth most prevalent form of cancer in men and women, respectively, it is now recognized as the fourth leading cause of cancer deaths in the United States. At the present time, gemcitabine is considered the drug of choice. This pyrimidine antimetabolite, 2',2'-difluorodeoxycytidine, an analog of deoxycytidine, has shown beneficial effects clinically in the treatment of pancreatic cancer (21). However, the primary benefit is in the palliation of disease symptoms and not increased patient survival. The addition of SM to the current treatment protocol may have an important clinical impact. In this report, we present data exploring the mechanism by which SM synergizes with gemcitabine to enhance apoptosis in pancreatic cancer. The data are consistent with the proposal that SM enhances the apoptotic response by increasing SM substrate available to drug-activated SMase, in effect priming the pathway for production of ceramide. Our results suggest this may be a general phenomenon for enhancement of the apoptotic response.

MATERIALS AND METHODS

Materials. Egg yolk SM was purchased from Avanti Polar Lipids (Alabaster, AL), gemcitabine (Gemzar) was purchased from Eli Lilly (Indianapolis, IN), MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was obtained from Promega (Madison, WI), fluorescein conjugated annexin-V was obtained from PharMingen (San Diego, CA), JC-1 dye was purchased from Molecular Probes (Eugene, OR), diacylglycerol kinase was purchased from BioMol (Plymouth Meeting, PA), and radiolabeled substrates was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were obtained from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI). RPMI media, containing 10% fetal calf serum, penicillin/streptomycin, sodium pyruvate, glutamine, and nonessential amino acids was used throughout.

Cell Growth and Viability Assay. Cells were seeded into the central 60 wells of a 96-well plate at a density of 2,000 cells/well in 100 μ L RPMI. The

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next day, drug and/or SM (in 100 μL of RPMI media) were added to the desired final concentration. After 4 days (~4 doublings), MTS reagent was added, the plates were incubated at 37°C for 4 hours, and the absorbance read at 490 nm. The percentage of viable cells was defined as A_{490} of gemcitabine and/or SM-treated cells divided by the A_{490} of cells receiving no treatment, multiplied by 100%. SM liposomes were prepared by suspending lipid in 0.9% sodium chloride at a concentration of 100 mg/mL and repeatedly passing the suspension through a warm Avanti Mini-Extruder with the 1 and 0.1 μm filters.

Apoptosis Measurements. Cells were seeded into 6-well plates at 2.5 to 5×10^4 cells per well, in triplicate. The next day, nonadhered cells were removed by gentle washing, and the media were replaced with fresh media containing SM and/or gemcitabine at the desired concentrations. Four days later, the cells were scraped into their own media, and the contents of individual wells, including detached cells, were centrifuged at $500 \times g$. The cells were resuspended in 200 μL PBS [20 mmol/L NaPO_4 (pH 7.6), 120 mmol/L NaCl, 2.7 mmol/L KCl] and split into two equal aliquots. In one tube, 2.5 μL of annexin-V-fluorescein conjugate (1:40 dilution) and 2.5 μL of a 50 $\mu\text{g}/\text{mL}$ solution of propidium iodide were added for determination of phosphatidylserine translocation to the outer leaflet of the plasma membrane and cell viability, respectively. The other aliquot received 2.5 μL of a 100 $\mu\text{g}/\text{mL}$ solution of JC-1 dye for the quantitation of intact, respiring mitochondria. After a 10-minute incubation at room temperature, the cellular fluorescence at 530 (FL1) and 585 nm (FL2) was measured in both tubes with a Becton-Dickenson FACSCaliber (BD Bioscience San Jose, CA). Cells displaying phosphatidylserine on their surface (positive annexin-V fluorescence) were considered to be apoptotic, regardless of viability (propidium iodide staining). Cells staining positive for propidium iodide uptake were considered dead, regardless of annexin-V staining. JC-1 fluoresces green in the cytoplasm and in depolarized mitochondria and red in actively respiring mitochondria. Increased cellular green fluorescence (FL1) was indicative of the loss of the mitochondrial membrane potential (MMP).

Protein Extraction and Analysis. Approximately 2.5 to 10×10^5 Panc1 cells were seeded per T175 flask. After allowing the cells to attach overnight, the media were removed, and 20 mL of fresh media containing various amounts of SM and/or 100 nmol/L gemcitabine were added. The cells were incubated for an additional 4 days (~4 doublings) and scraped into their own media. The contents from individual flasks were centrifuged at $500 \times g$ for 10 minutes at 4°C, and the cell pellets were resuspended in 5 mL PBS. For each flask, two 250- μL aliquots were transferred to flow cytometry tubes for apoptosis measurements (described above), 1 mL was transferred to a 15-mL tube containing 1 mL CHCl_3 and 2 mL methanol for lipid extraction, and the remainder was recentrifuged at $500 \times g$ for 10 minutes at 4°C to obtain a cell pellet for protein extraction (22).

Cell pellets were resuspended in 0.35 mL of lysis buffer that consisted of 250 mmol/L sucrose, 10 mmol/L HEPES (pH 7.2), 1 mmol/L EDTA, 1 mmol/L phenyl-methyl-sulfonyl-fluoride, and 1 $\mu\text{g}/\text{mL}$ of each protease inhibitor antipain, chymostatin, leupeptin, and pepstatin A. The samples were lysed by three 10-second bursts of 50 Watts, using a model W185 sonifier (Health System-Ultrasonics Inc., Plainview, NY), with 2-minute cooling intervals. The lysed cells were underlaid with 250 μL of lysis buffer containing 1.2 mol/L sucrose and then centrifuged at $3,000 \times g$ for 10 minutes at 4°C. The top 400 μL was recovered, diluted with lysis buffer without sucrose to bring the sucrose concentration to 250 mmol/L, and stored at -80°C until use. Protein concentration was approximated by spectrophotometry at 260 nm and 280 nm with the following formula: protein (mg/mL) = $1.56(A_{280}) - 0.764(A_{260})$. To quantitate neutral and acidic SMase activities, the release of [^3H]choline from [^3H -methyl]choline-SM into an aqueous soluble form under different pH conditions was measured (23). The conversion of UDP-[^3H]glucose to a *t*-butylmethyl ether soluble product, [^3H]glucosylceramide, was used to measure glucosylceramide synthase (GCS) activity (24).

Lipid Extraction and Analyses. The samples (1 mL cells, 1 mL CHCl_3 , and 2 mL methanol) were vortexed; an additional 1 mL of CHCl_3 was added and vortexed; 1 mL of deionized water was added and vortexed; and the aqueous and organic layers were separated by centrifugation at $3000 \times g$ (25). The lower organic layer was recovered and dried under N_2 in a clean tube. Total phosphate was determined by the method of Ames (26). Ceramide content was measured by the diacylglycerol kinase method and related to total cellular lipid as moles of ceramide per mole of phosphate.

Statistical Analyses. Student's *t* test was used to assess relatedness, with *P* values < 0.05 indicating statistically significant differences. For assessment of synergy, the combination index (CI) was determined by median effect analysis (27, 28). The equation used to calculate the combination index was $\text{CI} = (D_1/Dx_1) + (D_2/Dx_2) + (D_1D_2/Dx_1Dx_2)$, where D_x is the individual drug concentration at its respective IC_{50} and D is the concentration of drug in the combination that results in 50% growth inhibition. The subscripts 1 and 2 refer to different drugs, namely, gemcitabine and SM. A CI value < 0.9 indicates synergism, CI = 0.9 to 1.1 indicates additivity, and CI > 1.1 indicates antagonism.

RESULTS

SM Enhances Chemosensitivity in Pancreatic Cancer Cells. An initial study was done to examine the intrinsic toxicity of SM. By generation of a SM dose-response curve for Panc1 cells, the IC_{50} and IC_{10} , were shown to be 1.2 mg/mL and 0.2 mg/mL, respectively. Dose-response curves were then generated with gemcitabine given either alone or in combination with SM at its IC_{10} . The inclusion of SM with gemcitabine resulted in a dramatic increase in cytotoxicity (Fig. 1). The IC_{50} values for gemcitabine were 78.3 ± 13.7 nmol/L in the absence and 13.0 ± 3.0 nmol/L in the presence of SM, an approximate 6.0-fold increase in sensitivity for the combined treatment group ($P < 0.001$). The CI was 0.36, an indication of strong synergistic interaction between SM and gemcitabine in Panc1 cells. These studies were repeated with the AsPc1 human pancreatic cancer cell line with similar results (*i.e.*, the administration of SM with gemcitabine provided an enhanced antitumor effect over that observed with gemcitabine alone). With AsPc1, the IC_{50} and IC_{10} for SM given alone were 0.7 and 0.2 mg/mL, respectively. The IC_{50} values for gemcitabine were 30.5 ± 10.5 nmol/L in the absence versus 9.0 ± 2.1 in the presence of 0.2 mg/mL SM, a 3.1-fold increase in sensitivity ($P = 0.012$). The CI was 0.52, indicating a moderate synergistic interaction between SM and gemcitabine in this cell line.

Gemcitabine-Induced Loss of MMP and Increase in Apoptosis and Cell Death Is Enhanced by SM. Initial studies were done to determine the effects of SM given alone. Panc1 cells were grown with or without 0.2 mg/mL SM for a period of 4 days. Flow cytometry was then used to determine mitochondrial depolarization, a very early stage in apoptosis, as well as phosphatidylserine translocation to the outer plasma membrane leaflet, a middle stage in apoptosis. Incubation of the Panc1 cells with SM provided only a minor increase in the apoptotic indices (Table 1); the percentage of cells displaying loss of MMP increased from $4.2 \pm 3.2\%$ to $8.8 \pm 3.8\%$ ($P = 0.01$), and the percentage of apoptotic cells increased from $3.6 \pm 2.6\%$ to

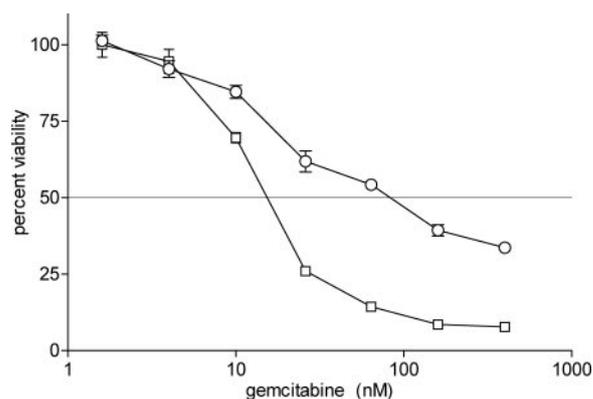


Fig. 1. SM enhances chemosensitivity of Panc1 cells to gemcitabine. The viability of Panc1 cells was examined as a function of gemcitabine concentration in the absence or presence of 0.2 mg/mL SM. The figure shows data generated from a single, representative experiment. \circ , no SM; \square , with 0.2 mg/mL SM.

Table 1 Sphingomyelin enhances gemcitabine-induced mitochondrial depolarization, apoptosis, cell death, and ceramide formation in Panc1 cells

Gemcitabine *	SM *	MMP %	Apoptosis %	Cell death %	pmol ceramide/nmole PO ₄
0	0	4.2 ± 3.2	3.6 ± 2.6	18.5 ± 7.0	0.46 ± 0.29
0	0.2mg/ml	8.8 ± 3.8	6.5 ± 3.8	23.7 ± 7.7	1.09 ± 0.42
100 nmol/L	0	25.9 ± 9.6	19.9 ± 12.9	46.0 ± 4.2	0.59 ± 0.34
100 nmol/L	0.2mg/ml	48.7 ± 7.4	42.1 ± 12.7	67.9 ± 9.7	1.58 ± 0.55

* Panc1 cells were treated with either 100 nmol/L gemcitabine and/or 0.2 mg/mL SM before measurement of MMP, apoptosis, cell death and ceramide.

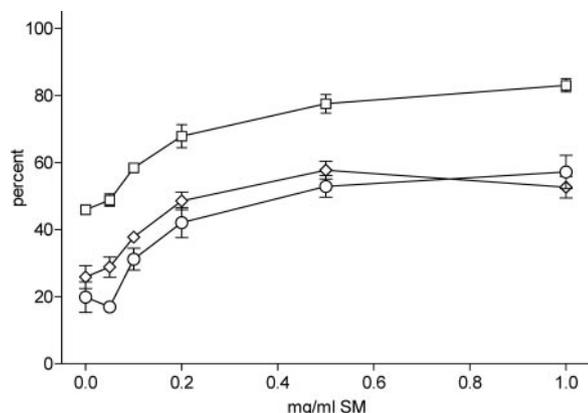


Fig. 2. SM enhances gemcitabine-induced mitochondrial depolarization, apoptosis, and cell death. At a constant gemcitabine concentration (100 nmol/L), MMP, apoptosis, and cell death were examined in the presence of increasing SM levels. ○, apoptotic; □, nonviable; ◇, depolarized mitochondria.

6.5 ± 3.8% ($P = 0.04$), each in the absence and presence of SM, respectively. The percentage of nonviable cells, with or without SM, was not significantly different (18.5 ± 7.0%) in the absence and (23.7 ± 7.7%) in the presence of SM, respectively ($P = 0.24$). It should be noted that when untreated cells were harvested on day 4, approximately 20% of the cells in each experiment were nonviable, predominantly through a nonapoptotic pathway. However, the percentage of cells which die, or enter into apoptosis, as a result of SM exposure was minimal, indicating that SM, by itself, was relatively nontoxic to Panc1 cells.

The ability of SM to amplify gemcitabine-induced apoptosis in Panc1 cells was examined by treating cells with 100 nmol/L gemcitabine in the presence of various concentrations of SM. Gemcitabine by itself is capable of inducing loss of MMP, apoptosis, and cell death. As such, a baseline cytotoxicity study was done to examine the effect of gemcitabine treatment on these parameters. Panc1 cells were grown either in the absence or presence of 100 nmol/L gemcitabine for 4 days. At this time point, the percentage of cells having depolarized mitochondria increased to 25.9 ± 9.6% ($P < 0.001$), the percentage of apoptotic cells increased to 19.9 ± 12.9% ($P < 0.001$), and the percentage of nonviable cells increased to 46.0 ± 4.2% ($P < 0.001$), as compared with the untreated cells noted above (Table 1; Fig. 2).

The combined treatment of Panc1 cells with SM and gemcitabine provided a statistically significant, greater than additive, enhancement of apoptosis and cell death. Furthermore, these parameters increased in a dose-dependent manner (range of P values, $P < 0.03$ to $P < 0.001$), with apparent saturation of the effect at SM concentrations above 0.2 mg/mL (Fig. 2). The addition of 0.2 mg/mL SM with 100 nmol/L gemcitabine resulted in the loss of MMP, apoptosis, and cell death in 48.6 ± 7.4% ($P < 0.001$), 42.1 ± 12.7% ($P < 0.002$), and 67.9 ± 9.7% ($P < 0.001$) of the cells, respectively, as compared with cells treated with gemcitabine alone (Table 1).

SM Enhances Gemcitabine-induced Production of Ceramide.

Panc1 cells were incubated with 100 nmol/L gemcitabine and various concentrations of SM for 4 days to determine whether the observed enhancement of apoptosis was associated with increased formation of

ceramide. Lipids were extracted from the cells, the ceramide was quantified, and the results were normalized to the total organic phosphate present in the sample. In the absence of SM, the ceramide content of cells treated with or without 100 nmol/L gemcitabine was not significantly different (0.59 ± 0.34 and 0.46 ± 0.29 pmol ceramide/nmol PO₄, respectively, $P > 0.1$; Table 1).

In the presence of increasing concentrations of SM, ceramide levels rose in both groups, with and without 100 nmol/L gemcitabine (Table 1; Fig. 3). In the absence of gemcitabine, cellular ceramide levels rose from untreated control levels to 0.90 ± 0.28 ($P = 0.015$), 1.09 ± 0.42 ($P = 0.01$), and 1.02 ± 0.40 ($P = 0.009$) pmol ceramide/nmol PO₄ at 0.05, 0.2, and 0.5 mg/mL SM, respectively. In the presence of 100 nmol/L gemcitabine, cellular ceramide levels rose from gemcitabine-treated base levels to 0.87 ± 0.51 ($P = 0.11$), 1.58 ± 0.55 ($P < 0.001$) and 2.15 ± 0.50 ($P < 0.001$) pmol ceramide/nmol PO₄ at 0.05, 0.2, and 0.5 mg/mL SM, respectively.

Importantly, whereas ceramide levels increased significantly in all groups receiving SM, ceramide increased to a greater extent in the groups that also received gemcitabine compared with cells that received SM alone. Statistical significance was observed at 0.2 and 0.5 mg/mL SM ($P = 0.038$ and $P < 0.001$, respectively) for the comparison of cells with and without 100 nmol/L gemcitabine. Furthermore, the observed increases in ceramide levels were greater than additive at these concentrations of SM.

Acidic SMase Is Activated by Gemcitabine in Panc1 Cells. Both acidic and neutral SMases have been implicated in the generation of ceramide in response to environmental stresses. Because the involvement of one or the other SMase has been noted to be cell line dependent, we measured both acidic and neutral SMases in Panc1 cell extracts. Panc1 extracts showed a 1.8-fold increase in acidic SMase activity in the presence of gemcitabine relative to untreated cells (34.8 ± 4.1 versus 19.0 ± 3.6 nmol choline released/mg protein, respectively, $P < 0.001$; Fig. 4A). No significant differences between cells treated with or without SM were observed ($P > 0.05$), and this was independent of gemcitabine exposure. These data indicate that SM, by itself, does not induce, nor does it alter the degree to which

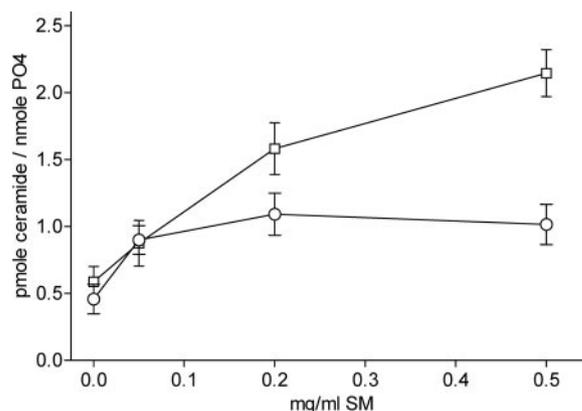


Fig. 3. SM enhances gemcitabine-induced formation of ceramide. Ceramide levels were determined by the diacylglycerol kinase method and were normalized to the amount of lipid phosphate. ○, no gemcitabine; □, 100 nmol/L gemcitabine.

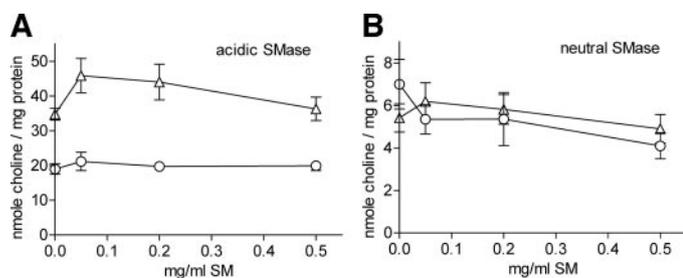


Fig. 4. Gemcitabine induces acidic, but not neutral, SMase activity. Panc1 cellular extracts were analyzed for SMase activities. The acidic and neutral SMase activities (A and B, respectively) were determined (pH 5, acidic or pH 7.4 neutral). ○, no gemcitabine; △, 100 nmol/L gemcitabine.

gemcitabine induces, activation of acidic SMase. In contrast, neutral SMase was not activated by either gemcitabine or SM (Fig. 4B).

Glycosylceramide Synthase Activity Is Unaffected by Either Gemcitabine or SM. GCS has emerged as a potential mediator of multidrug resistance through its action on ceramide. Cabot *et al.* (29) have examined modulation of GCS expression and found that increasing GCS activity leads to multidrug resistance (MDR), as would be expected if many drugs use the ceramide-mediated apoptotic pathway, whereas decreasing the activity leads to a build up of ceramide and enhanced chemosensitivity (30). The activity of GCS was measured in extracts of Panc1 cells exposed to gemcitabine and/or SM (Fig. 5). It was found that the addition of gemcitabine did not induce a change in GCS activity ($P > 0.1$). In the absence of gemcitabine, SM alone induced a weak decrease in activity at 0.05 and 0.2 mg/mL SM ($P = 0.05$ and 0.03, respectively) but was without significant effect in the presence of gemcitabine ($P > 0.1$).

DISCUSSION

Cancer cells are inherently resistant to apoptotic stimuli. In many instances this includes resistance to apoptosis-inducing chemotherapeutic agents (1, 31–33). However, it is important to note that cancer cells can, and do, die via the apoptotic pathway. This implies that cancer cells possess functional apoptotic machinery but that the signal(s) required to activate the pathway are either not being generated or not being recognized.

We have postulated that at least one of possibly many mechanisms by which cancer cells become resistant to apoptosis is through attenuation of ceramide production. Ceramide, a second messenger in the apoptosis pathway, is often generated after exposure to chemotherapeutic agents. It is clear that ceramide, sphingosine, and sphingosine-1-phosphate are potent, bioactive sphingolipids (3, 4, 34) and that modulating the intracellular levels of these lipids can lead to either cell growth or death (35). These observations have led some to hypothesize a sphingolipid rheostat that determines whether the cell undergoes mitosis or apoptosis (36). Consequently, all enzymes that affect ceramide and sphingosine metabolism are potential regulators of cell growth and/or cell death.

In addition to modulation of intracellular ceramide levels through biosynthetic and catabolic mechanisms, the availability of SM substrate may also play a role in the amplitude of ceramide generation. Evidence suggests that not all cellular SM is equivalent with regards to its potential to be converted to proapoptotic ceramide, and that the intracellular site of ceramide production is critical (5, 8, 11). This opens the possibility that depletion of specific intracellular pools of signaling SM, as opposed to the bulk of cellular SM, could lead to increased chemoresistance, whereas replenishment of these pools could lead to enhanced drug sensitivity. In other words, the limiting

step for the induction of apoptosis in cancer cells is the availability of SM substrate for the drug-activated SMases.

It is important to note that in our model, chemosensitivity of normal cells would not increase through the addition of SM. We hypothesize that in normal cells, the SM signaling pools are already sufficiently filled so as to not limit the production of ceramide. Instead, the limiting factor in the production of ceramide is the availability of activated SMase. This corollary is supported by our observation that SM, when administered at levels of up to 10 mg/day for 7 days to mice, did not induce toxicity (18).

SM biosynthesis is compartmentalized, beginning on the cytosolic face of the endoplasmic reticulum with the formation of ceramide (37) and finishing in the cisternae of the golgi with SM synthase-mediated transfer of a phosphocholine head group from phosphatidylcholine to ceramide (38). Approximately 40 to 90% of cellular SM is present within the exoplasmic face of the plasma membrane (39). However, various amounts of SM have been reported in the endosomes, inner leaflet of the plasma membrane, golgi, nucleus, and mitochondria.

Activation of SMase is the predominant pathway for the generation of ceramide in response to chemotherapeutic agents (3, 11, 12). Acidic SMase is located primarily within the lysosomes, but also in the caveolae, the sphingolipid rich microdomains of the plasma membrane that are involved in receptor-mediated signaling (13). Several neutral SMase enzymes have been identified, including plasma membrane-bound, Mg^{2+} -dependent and cytosolic, Mg^{2+} -independent enzymes, as well as incompletely characterized nuclear and mitochondrial forms (2, 14–17).

As noted, the intracellular site of ceramide synthesis is critical to the apoptotic response (5, 8, 11). Whereas extracellularly applied bacterial SMase readily cleaves outer leaflet plasma membrane SM to ceramide, it does not lead to an apoptotic response, whereas intracellular SMase expression does (13, 40). It was suggested that caveolar bound SMase, or inner leaflet associated SMase, is responsible for generating ceramide (41). However, more recent studies point to the mitochondria as the site of ceramide generation and action (2, 42, 43). In mitochondria, ceramide-induced apoptosis could be blocked by bcl-2, indicating that ceramide production is upstream of the bcl-2 control point (42). Whereas the site of ceramide production and action is not firmly established, the sum of the studies strongly suggest that distinct intracellular pools of SM exist for the purpose of signaling the initiation of an apoptotic response. Thus, modulation of the sphingolipid content within these pools can influence the chemosensitivity of cells.

The most straightforward approach to examine the impact of SM

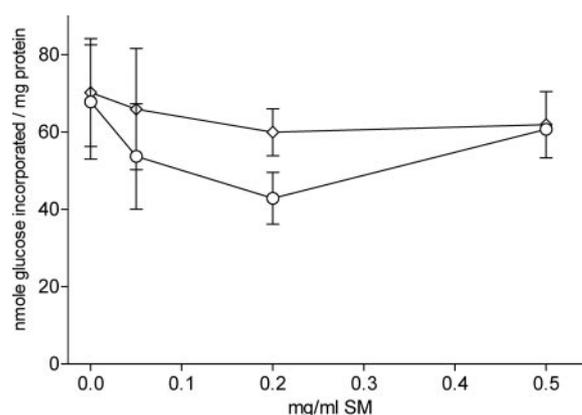


Fig. 5. Glycosylceramide synthase activity is not affected by either gemcitabine or SM. Panc1 cellular extracts were analyzed for glycosylceramide synthase activity by monitoring the transfer of glucose from [3H -glucose]UDP to ceramide. ○, no gemcitabine; ◇, 100 nmol/L gemcitabine.

levels on chemosensitivity is to apply exogenous SM to cells in culture and determine the level of drug sensitivity/resistance. We have tested this approach with colonic tumor cells and found that four of seven cell lines had greater sensitivity to both 5-FU and Adriamycin in the presence of exogenous SM (19). Three of these cell lines grown as xenografts in athymic nude mice showed greater sensitivity to 5-FU coadministered with SM, as compared with 5-FU given by itself (18).

Our current findings are consistent with the hypothesis that it is possible to enhance chemosensitivity through supplementation of SM substrate pools with exogenous SM, in effect priming the cellular machinery responsible for generating proapoptotic ceramide. As we showed with colonic cell lines, we found that both AsPc1 and Panc1 cells became more sensitive to chemotherapy, in this case gemcitabine, the front-line drug for pancreatic cancer, when given in conjunction with nontoxic doses of SM. Increased chemosensitivity was associated with an increased mitochondrial depolarization, apoptosis, and cell death. It is important to note that SM, when administered as a single agent, did not appreciably increase any of these three parameters, and the effects of gemcitabine, when administered alone, were modest at the concentrations tested. In contrast, the combination of gemcitabine and SM provided an enhanced apoptotic response, leading to increased cell death.

Currently, there is some controversy over which SMase is responsible for generating ceramide during the apoptotic response (reviewed in ref. 44). In the current studies, it was determined that acidic SMase was activated after a 4-day exposure to gemcitabine, whereas neutral SMase activity remained unchanged. In contrast, some investigators have shown early activation of neutral SMase (44). Thus, at this time, we cannot exclude the possibility that neutral SMase is involved or responsible for generating the ceramide signal or that ceramide formation is exclusively attributable to the action of SMase(s).

Our observations of ceramide levels, with respect to the induction of apoptosis and the reduction of cell viability, further support the premise that all cellular SM is not equivalent. Specifically, we note that 0.2 mg/mL SM alone resulted in an approximate 250% increase in cellular ceramide levels compared with untreated cells. However, this was not translated into a significant increase in apoptosis or cell death. In other words, this ceramide is nonapoptotic in that it does not activate apoptosis. This is consistent with our previous *in vivo* findings indicating that SM is nontoxic and does not have significant antitumor effects by itself. Evidently, ceramide generated in the absence of chemotherapeutic stimuli does not have access to effector molecules responsible for the initiation of apoptosis.

Gemcitabine at 100 nmol/L, in the absence of exogenous SM, did not significantly alter intracellular ceramide levels, compared with ceramide levels in untreated cells, but provided a modest increase in apoptosis with consequent loss of cell viability. That we did not observe an increase in ceramide levels after administration of gemcitabine, in the absence of SM, was somewhat surprising. However, it is possible that the ceramide, once used to initiate apoptosis, is metabolized before the cells are harvested on day 4 post-treatment. In this context, the inclusion of SM might sustain the levels of ceramide by providing for continuous replenishment of substrate or by inhibiting the conversion of ceramide to other sphingolipids.

The sum of the data presented here is consistent with the model that tumor cells are intrinsically resistant to chemotherapy, in part because of a reduced SM signaling pool. Because of this, induction of apoptosis is diminished. In the present study, supplementation of the SM signaling pool via the application of exogenous SM enhanced the levels of gemcitabine-induced formation of ceramide, mitochondrial depolarization, apoptosis, and cell death. These results agree with other research groups who have found that modulation of sphingolipid metabolism can have profound effects on cell viability and drug

resistance (reviewed in ref. 45). SM seems to interact in a synergistic manner with agents that induce apoptosis, thereby enhancing their antitumor effects. This result has been observed in several tumor types, including pancreatic, colorectal (18, 19), breast (46), and lymphoma (46), and appears to be effective with several chemotherapeutic agents, as well as with radiation (18–20, 46). Because SM is nontoxic, the use of SM to maximize the apoptotic potential of tumoricidal agents could have broad applicability in the development of more effective treatment procedures.

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Synergistic Interaction between Sphingomyelin and Gemcitabine Potentiates Ceramide-Mediated Apoptosis in Pancreatic Cancer

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