

Ceramide Regulates Gemcitabine-Induced Senescence and Apoptosis in Human Pancreatic Cancer Cell Lines

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Abstract

Bioactive sphingolipids are potent intracellular signaling molecules having profound effects on cell death, growth, and differentiation. Pharmacologic manipulation of sphingolipid levels could have a significant effect on the induction of apoptosis by anticancer agents, and thus, improve treatment efficacy. We observed that gemcitabine cannot completely kill AsPc1 and Panc1 human pancreatic cancer cells in culture; even at high concentrations of gemcitabine, 30% to 40% of the cells remain viable. By adding sphingomyelin to the culture medium, gemcitabine-induced cell death increased synergistically to >90%. Panc1 cells that survived high concentrations of gemcitabine had an increase in β -galactosidase activity, a marker of senescence. The inclusion of sphingomyelin with gemcitabine reduced β -galactosidase activity, as compared with cells treated with gemcitabine alone. Expression of p21^{waf1/cip1} in both cell lines exposed to sphingomyelin, gemcitabine, and gemcitabine + sphingomyelin varied relative to the untreated group. C₈-ceramide induced both cell death and senescence in a dose-dependent manner. These results indicate that gemcitabine induces senescence in pancreatic cancer cells and that sphingomyelin-enhanced chemosensitivity is achieved through reducing the induction of senescence by redirecting the cell to enter the apoptotic pathway. Ceramide levels seem to be critical to this decision, with cell cycle progression being uninhibited at low ceramide levels, senescence induced at moderate levels, and apoptosis initiated at high levels. Our results provide further evidence that targeting the sphingolipid metabolism is a means of enhancing the efficacy of chemotherapeutic agents. (Mol Cancer Res 2009;7(6):890–6)

Introduction

Pancreatic carcinoma provides a major challenge both in terms of diagnosis and subsequent treatment options. It is one of the most insidious forms of cancer with a 5-year survival rate of <5%. The frontline chemotherapeutic agent for nonresectable disease is gemcitabine (2',2'-difluoro-2'-deoxycytidine; refs. 1, 2). Although this drug has achieved a modest increase

in survival (median duration of survival for gemcitabine-treated patients was reported to be 5.65 months compared with 4.41 months for 5-fluorouracil-treated patients; ref. 3), its main clinical value is in the palliation of disease symptoms and improved quality of life. Thus, an understanding of the mechanism(s) behind gemcitabine resistance in pancreatic carcinoma may lead to improved efficacy.

Cancer cells undergo apoptosis in response to a variety of stimuli and use sphingolipids as intracellular second messengers. The balance between the relative levels of the bioactive sphingolipids, ceramide, sphingosine, and sphingosine-1-phosphate, has been proposed to act as a "rheostat" to determine the fate of the cell with respect to survival (4, 5). Proapoptotic ceramide is generated by increased *de novo* synthesis, or more commonly, through hydrolysis of sphingomyelin (SM). Sphingomyelinases (SMase) are activated by a variety of stresses, including chemotherapeutics. On the other hand, expression of ceramide-catabolizing (transforming) enzymes (e.g., ceramidase and glucosylceramide transferase) leads to chemoresistance. Thus, pharmacologic manipulation of sphingolipid synthesis and/or degradation could have a significant effect on the induction of apoptosis by anticancer drugs.

We have shown that the inclusion of sphingomyelin in the cell culture medium increased the chemosensitivity of Panc1 human pancreatic carcinoma cells to gemcitabine (6), and that the addition of sphingomyelin to gemcitabine treatment of Panc1 tumor xenografts in severe combined immunodeficient mice results in a significant increase in animal survival time compared with single-agent therapy.¹ We also noted that, even at high concentrations, gemcitabine was unable to completely kill the cells, with viability remaining at ~30% to 40%. In this report, we investigated the nature of these remaining viable cells because they represent a population from which the tumor could potentially regrow or a population that might be able to support the growth of tumor cells.

In normal cells, senescence is brought about by the gradual shortening of the cells' telomeres and is functionally defined by irreversible growth arrest and increased levels of cell cycle-inhibitory proteins (e.g., p53, p21, and Rb; ref. 7). Senescent cells are viable, but mitotically inactive, and are characterized by the expression of the enzyme, β -galactosidase. In cancer cells, senescence can be induced by at least two distinct mechanisms (8). In the first, "replicative senescence", inhibition of telomerase leads to a phenotype closely resembling that of normal cells and occurs after several cell divisions. In the second, "senescent-like state", senescence is induced through the

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overexpression of cell cycle-inhibitory proteins or DNA damage. This latter form is initially reversible, but proceeds to irreversible senescence through unknown mechanism(s). Senescent cells present a barrier to the effective treatment of cancer because they might be capable of subsequently re-entering active cell cycling or could provide support to other cancer cells, such as cancer stem cells, through the release of pro-growth and pro-angiogenic factors. Thus, eliminating or preventing the emergence of senescent cancer cells could be an important element in improving tumor response.

In this work, we show that *in vitro* treatment of pancreatic carcinoma cells with gemcitabine induces senescence. When sphingomyelin is provided in the culture medium, a significant reduction of senescence is observed with concomitantly enhanced cell death. Short-chain ceramide (C_8 -ceramide) was also capable, in a dose-dependent manner, of inducing both senescence and apoptosis. C_8 -ceramide-induced senescence occurred at lower concentrations, whereas apoptosis was observed at higher concentrations. These data lead us to propose a model in which sphingolipids play a role in the decision between cell cycle progression, senescence, and apoptosis.

Results

Exogenous Sphingomyelin Enhances Chemotherapy-Induced Gemcitabine Cytotoxicity

We investigated the sensitivity of human pancreatic carcinoma cell lines to gemcitabine in culture, and observed a significant reduction in viability at clinically relevant concentrations (9). However, the minimum viability of AsPc1 and Panc1 that could be attained by exposure to gemcitabine alone was 35% and 40%, respectively, as measured using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) cell viability assay (Fig. 1). The inclusion of sphingomyelin in the medium (at the maximum concentration which results in <10% cell death as a single agent) resulted in >90% reduction in viability and a reduction in the gemcitabine IC_{50} for both cell lines. The reduction was statistically significant for AsPc1 ($IC_{50} = 30.5 \pm 10.5$ and 9.0 ± 2.1 nmol/L in the absence and presence of sphingomyelin, respectively; $P = 0.012$) and Panc1 cells ($IC_{50} = 52.3 \pm 13.6$ and 3.27 ± 2.22 nmol/L in the absence and presence of sphingomyelin, respectively; $P < 0.001$). For each cell line, the sphingomyelin-mediated reduction in the gemcitabine

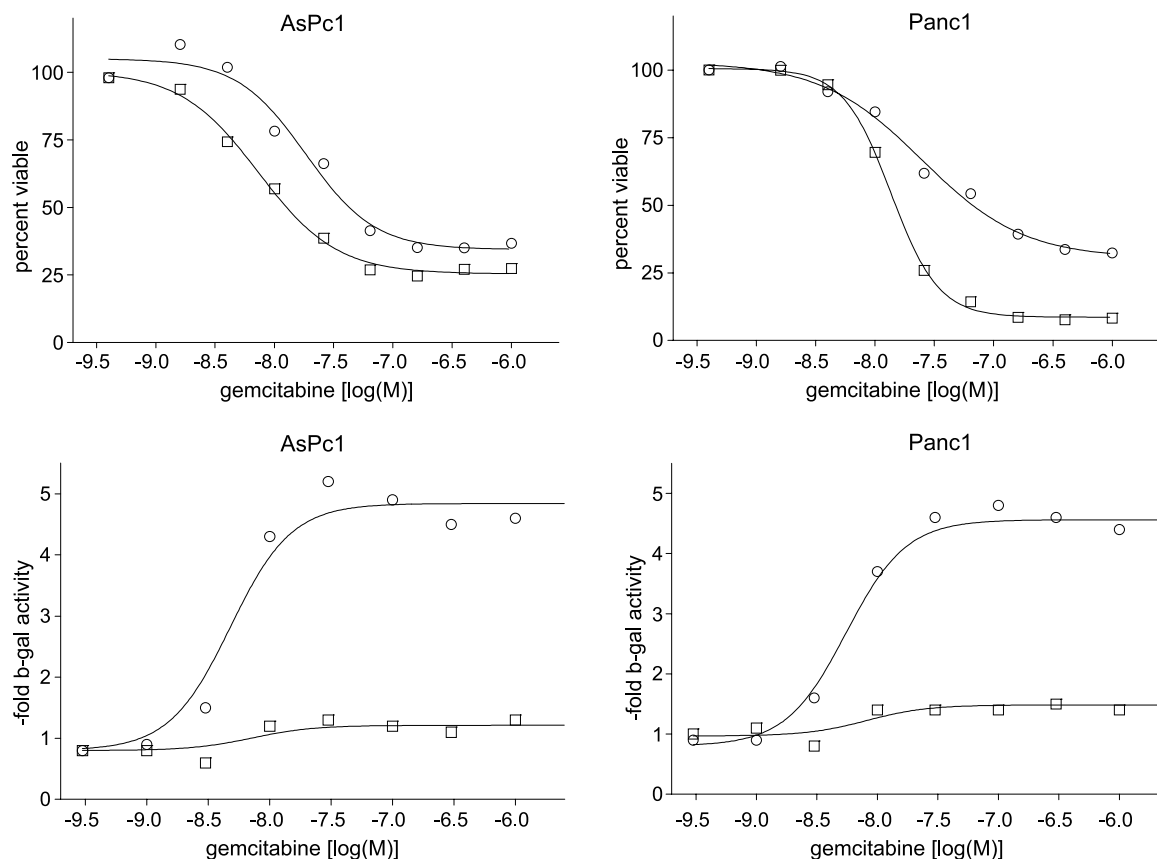


FIGURE 1. Sphingomyelin interacts synergistically with gemcitabine to enhance chemosensitivity and suppress the induction of senescence. Top, cell viability (as determined by use of the MTT reagent); circles, no sphingomyelin added; squares, sphingomyelin included. Bottom, β -galactosidase activity (as determined using C_{12} -FDG); circles, no sphingomyelin added; squares, sphingomyelin included. Where indicated, sphingomyelin was included at 0.1 mg/mL (AsPc1) or 0.2 mg/mL (Panc1). In each case, the amount of sphingomyelin used was the maximum attainable level that resulted in <10% cell death when provided as a single agent.

Table 1. Modulators of Sphingolipid Metabolism Affect Gemcitabine Cytotoxicity in Panc1 Cells

Modulator*	Target	Cell Death with Modulator Alone (%)	Gemcitabine, IC ₅₀ (nmol/L)	Sensitization Factor [†]	CI [†]
None (gemcitabine alone)	—	—	52.3 ± 13.55	—	—
Synergistic reagents					
Sphingomyelin (0.2 mg/mL)	—	<10	3.68 ± 2.75	18.1*	0.16
Me-SM (1 μmol/L)	nSMase	40	10.9 ± 9.8	4.80*	0.41
PPMP (2 μmol/L)	UGCG	<10	14.4 ± 15.4	3.63*	0.48
MAPP (5 μmol/L)	Ceramidase	<10	13.2 ± 15.6	3.96*	0.41
Antagonistic reagents					
Imipramine (10 μmol/L)	aSMase	35	57.4 ± 31.1	0.91	1.68

Abbreviations: Me-SM, 3-*O*-methylsphingomyelin; PPMP, *DL*-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; MAPP, 1*S*,2*R*-*D*-erythro-2-(*N*-mristoylamino)-1-phenyl-1-propanol.

*The IC₅₀ values for the modulator alone are sphingomyelin, 1.95 ± 0.84 mg/mL; imipramine, 24.2 ± 9.60 μmol/L; MAPP, 30.0 ± 11.2 μmol/L; Me-SM, 4.89 ± 1.93 μmol/L; and PPMP, 9.56 ± 7.19 μmol/L. The concentration in parentheses was the amount used for determining the gemcitabine IC₅₀ value in the presence of modulator.

[†]The values for sensitization factor (fold change) and CI were calculated as in Materials and Methods. *, *P* < 0.05 for the comparison of gemcitabine vs gemcitabine + inhibitor IC₅₀ values.

IC₅₀ resulted from a synergistic interaction between gemcitabine and sphingomyelin [combination index (CI), 0.52 and 0.16, respectively].

The use of inhibitors of sphingolipid metabolism supports the concept that this pathway is involved in chemosensitization by exogenous sphingomyelin (Table 1). Inhibitors of acidic SMase were antagonistic to gemcitabine-induced apoptosis, whereas inhibitors of neutral SMase, glucosylceramide transferase and ceramidase, were synergistic with gemcitabine for tumoricidal activity.

Exogenous Sphingomyelin Reduces Gemcitabine-Mediated Cell Cycle Arrest and Senescence

As determined by microscopy and flow cytometry, the cells that survived exposure to gemcitabine were much larger than untreated cells (data not shown), suggesting that they might have undergone senescence. To investigate the effects of gemcitabine treatment on the cell cycle and induction of senescence, we measured the cell cycle distribution after exposing Panc1 cells to varying levels of gemcitabine and/or 0.2 mg/mL of sphingomyelin. Compared with untreated cells, treatment with sphingomyelin alone had no effect on cell cycle distribution. This was in contrast to 100 nmol/L of gemcitabine, which alone blocked cell cycle progression through S phase and resulted in the accumulation of a significant sub-G₁ population (i.e., apoptotic cells; Table 2). With the addition of sphingomyelin to gemcitabine, the sub-G₁ population increased further at the expense of all other populations.

Table 2. Effects of Gemcitabine and Sphingomyelin Cell Cycle Distribution

	Sub-G ₁	G ₁ -G ₀	S	G ₂ -M
Untreated	15.5	42	18.4	18.7
Sphingomyelin alone	14.3	39.8	17.4	22.1
Gemcitabine alone	44.9	28.8	17.5	6.4
Gemcitabine + sphingomyelin	57.4	23.6	12.9	4.3

NOTE: Units are percentages of gated cells.

β-Galactosidase activity is a commonly used marker of senescence (10). To measure β-galactosidase activity in individual cells by flow cytometry, cells were treated with gemcitabine and/or sphingomyelin in the presence of 5 μmol/L of dodecanoyl-fluorescein digalactoside (C₁₂-FDG), a fluorescent, lipophilic β-galactosidase substrate. We observed a large, dose-dependent increase in the activity of β-galactosidase in AsPc1 and Panc1 cells treated with gemcitabine alone as compared with untreated cells (Fig. 1). In cells treated with gemcitabine in the presence of sphingomyelin, β-galactosidase activity remained essentially unchanged compared with the no treatment and sphingomyelin alone groups. These data strongly suggest that gemcitabine blocked cell cycle progression and induced senescence, whereas supplementation of gemcitabine therapy with sphingomyelin resulted in significantly less induction of senescent-like cells concomitant with increased levels of cell death.

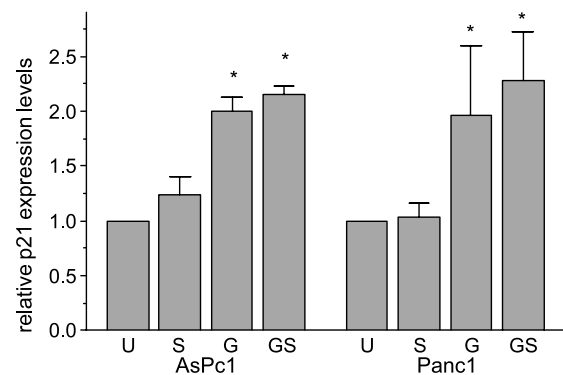


FIGURE 2. Cell cycle regulatory protein levels fluctuate with treatment. Approximately 10⁶ cells were treated with an IC₅₀ amount of gemcitabine and/or low, nontoxic levels of sphingomyelin [50 nmol/L gemcitabine ± 0.1 mg/mL sphingomyelin (AsPc1) or 100 nmol/L gemcitabine ± 0.2 mg/mL sphingomyelin (Panc1)]. P21^{waf1/cip1} protein levels were determined by Western blotting. The images were digitized and analyzed with the Un-Scan-It program. Expression levels were normalized to actin levels, then graphically depicted relative to protein levels in untreated cells (with SD shown). Asterisks, statistically significant differences between that group and the untreated cells (*P* < 0.05 for the gemcitabine alone or gemcitabine + sphingomyelin combination compared with the untreated or sphingomyelin alone groups).

Increased expression of the cell cycle regulatory protein p21^{waf1/cip1} has been noted in senescent cells. We examined p21^{waf1/cip1} in each of the cell lines after exposure to gemcitabine at the IC₅₀ with or without the addition of sphingomyelin at the maximum nontoxic dose (Fig. 2). We observed that sphingomyelin alone had no effect on p21^{waf1/cip1} levels in the cell lines, whereas gemcitabine alone increased the levels of this protein. The combination of gemcitabine and sphingomyelin resulted in the same change in protein levels as seen with gemcitabine alone, again demonstrating that sphingomyelin has no effect on protein levels. These results are consistent with the induction of senescence as a result of gemcitabine exposure.

Senescence and Apoptosis are Differentially Induced by Varying Levels of Ceramide

Our data suggest that increasing the intracellular ceramide level via greater conversion of sphingomyelin to ceramide leads to increased apoptosis at the expense of drug-induced senescence. If varying intracellular levels of ceramide lead to alternative responses, then the prevalence of apoptotic and senescent cells should show a ceramide dose-dependency. To test this, we treated cells with varying amounts of C₈-ceramide and examined viability by flow cytometry and senescence induction by β -galactosidase activity. The results showed that induction of both cell death and senescence was dependent on C₈-ceramide concentration in the medium (Fig. 3). The IC₅₀ for C₈-ceramide in Panc1 cells was 43.6 \pm 2.5 μ mol/L, with little effect on viability below 30 μ mol/L and near complete killing above 50 μ mol/L, as measured by propidium iodide exclusion. β -Galactosidase activity increased with the ceramide concentration, peaking at 21.7 \pm 1.5 μ mol/L and decreasing rapidly above this level. Similar results were seen with AsPc1 (maximum β -galactosidase activity at 20.0 \pm 5.7 μ mol/L; IC₅₀ = 42.1 \pm 1.9 μ mol/L). The dose-response curves of β -galactosidase activity and induction of cell death were only partially overlapping for each cell line and indicated that senescence occurs at lower ceramide concentrations than that needed for cell death. Qualitatively, the data for AsPc1 and Panc1 cells were very similar with respect to magnitude of β -galactosidase activity induction (~300% of the minimum level noted in the experiment), and a difference in C₈-ceramide concentration between the peak induction of β -galactosidase activity and cell death (~22 μ mol/L). These data are consistent with those of Venable et al., who found that 10 to 15 μ mol/L of C₆-ceramide also induced senescence in WI-38 normal diploid fibroblasts (11). These data suggest that AsPc1 and Panc1 share a mode of gemcitabine resistance which includes the ability to enter senescence, and that ceramide is a key signaling molecule for both cell death and senescence.

Discussion

Pancreatic cancer is one of the most chemotherapy-resistant cancers, with the frontline chemotherapeutic agent, gemcitabine, providing only a marginal increase in median survival (3). Drug resistance in cancer is complex and multiple pathways, often operating concurrently, contribute to overall resistance. One contributor to general drug resistance is through the specific regulation of intracellular ceramide levels (12). This

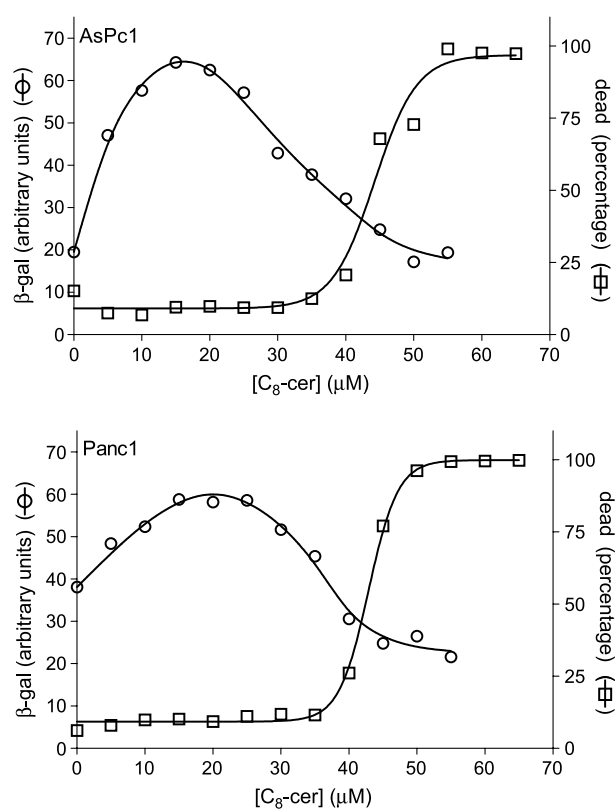


FIGURE 3. Ceramide-induced senescence occurs at lower concentrations than apoptosis. AsPc1 and Panc1 cells were seeded into 24-well plates and the effect of adding C₈-ceramide to the medium on β -galactosidase activity and viability was determined using C₁₂-FDG and propidium iodide, respectively. Representative results of three independent experiments. Squares, cell death; circles, β -galactosidase activity.

bioactive sphingolipid is a second messenger of cellular stress which, at high levels, leads to apoptosis. In the present study, we explored our observation that, even at very high concentrations of gemcitabine, a large fraction of human pancreatic carcinoma cells do not undergo cell death. We hypothesized that cell survival at high concentrations of gemcitabine is a reflection of the inability of the cells to produce ceramide at levels necessary to induce apoptosis. However, the ceramide levels reached would be sufficient to cause the cells to enter senescence and would account for the apparent reduction in cell viability noted in MTT assays. We further explored the possibility that the ability of cells to produce ceramide could be enhanced by supplementing the medium with sphingomyelin, and that this would lead to increased gemcitabine-induced cell death.

Both endogenous and exogenous sphingolipids have been shown to affect progression through the cell cycle. Several studies have shown that ceramide levels increase during G₁ and then decrease after the G₁-S restriction point (13, 14). In addition, neutral SMase has been implicated in ceramide production during cell cycle arrest (15). In confluent MCF7 cells, neutral SMase moved from the cytosol to the plasma membrane, with a concomitant increase in long-chain ceramides and senescence induction. Senescent cells have also been found to have altered sphingolipid metabolisms, with increased glycosylceramide

production at the expense of sphingomyelin (16). In WI-38 human diploid fibroblasts (11, 17), Molt-4 human leukemia (18) and A549 human lung carcinoma (19, 20), exogenous C₆-ceramide was able to block cell cycle progression in the G₀-G₁ phase. These data point to ceramide levels, particularly at the restriction point, being a major influence on whether cell cycle progression (very low ceramide), senescence, or apoptosis (high ceramide) occurs.

Bieberich has proposed that ceramide levels govern cell cycle progression and cell death, and are determined primarily by a balance between *de novo* synthesis and ceramide glucosylation (21). Because the sphingolipid metabolism is complex, we believe that this model may need modification in order to accommodate other factors affecting ceramide levels (Fig. 4). Potentially significant contributions to intracellular ceramide levels can be made by SMases and other ceramide-utilizing enzymes. This may be of special interest in the development of new means to treat cancer in which drug-activated SMases may provide for greater sensitivity of the tumor cells to induction of apoptosis by other agents. It has also been shown that subcellular compartmentalization of ceramide production, such as in the nucleus versus the mitochondria versus plasma membrane versus endoplasmic reticulum, has a role in the biological effect of ceramide production (12, 22, 23). Thus, intracellular ceramide levels are controlled by a complex integration of biosynthetic/catabolic, spatial, and temporal mechanisms which can lead to several alternative biological outcomes. We are currently examining the role of sphingolipid subcellular distribution and molecular identity on senescence and apoptosis induction in pancreatic cancer cell lines.

The mechanism by which sphingomyelin increases chemosensitivity is unclear. We found that sphingomyelin had no effect on the levels of p21^{waf1/cip1}, but instead, this protein was responsive to gemcitabine treatment. It remains possible that whereas total protein levels do not change with exposure to exogenous sphingomyelin, a more extensive analysis of pro-

tein function (e.g., response over time, subcellular localization, activation status, etc.) might reveal meaningful differences. Alternatively, the cells may respond to gemcitabine treatment by up-regulating p21 expression as a general initial response, separate and independent of sphingolipid levels, and initiate apoptosis only if ceramide levels subsequently increase. Future work will examine the functional status of apoptosis and cell cycle regulatory proteins on the induction of senescence and apoptosis in pancreatic carcinoma cell lines.

We suggest the model illustrated in Fig. 4, in which intracellular ceramide levels control the decision of whether to continue with the cell cycle, enter a senescent state, or undergo apoptosis. In this model, ceramide levels increase during G₁ as part of the normal progression through the cell cycle. At the restriction point, low levels of ceramide do not effectively inhibit the cell cycle, and subsequent continuation of the cycle leads to a reduction in ceramide levels. Moderate ceramide levels, such as that generated in response to stress, arrest the cell cycle and direct the cell to enter senescence. High ceramide levels also arrest the cell cycle, but bring about the induction of apoptosis in these cells. Given that gemcitabine activates multiple cell cycle control points, the restriction point is not necessarily the point at which the senescence versus apoptosis decision is made.

Figure 4 also indicates how sphingomyelin might influence this decision. We speculate that a limiting factor in ceramide production might be the availability of an adequate sphingomyelin pool to be used as a substrate for gemcitabine-activated acidic SMase (6). By increasing sphingomyelin availability, greater intracellular ceramide levels were generated in our cell lines, which led to increased apoptosis. In the treatment of Panc1 tumor xenografts in severe combined immunodeficient mice, we showed a reduced rate of tumor growth in animals receiving the combination of gemcitabine + sphingomyelin as compared with all other treatment groups ($P < 0.01$; median survival, 38, 39, 40, and 55 days for groups administered no treatment, sphingomyelin alone, gemcitabine alone, or gemcitabine + sphingomyelin, respectively).¹ Other agents and therapies that increase the levels of intracellular ceramide would also be expected to enhance gemcitabine toxicity, as was observed in our studies using sphingolipid metabolism inhibitors (Table 1). This latter point is in agreement with data from other laboratories that have examined the combination of modulators of sphingolipid metabolism with chemotherapeutics in a variety of tumor models (12).

In summary, the current studies and our previous work quantitating the levels of ceramide after gemcitabine and/or sphingomyelin in Panc1 cells (24) suggest the following series of events in Panc1 and AsPc1 cells: gemcitabine exposure activates (acidic) SMase, which is able to convert a limited amount of sphingomyelin into ceramide. Ceramide, at this concentration, is insufficient to induce apoptosis but is able to divert the cell into a senescent-like state. The inclusion of sphingomyelin allows for gemcitabine-activated SMase to produce sufficient quantities of ceramide to cause the cell to undergo apoptosis. We suggest that the manipulation of sphingolipid levels may prove useful in enhancing drug efficacy by eliminating senescent tumor cells that support the growth of the tumor with concomitant enhancement of the apoptotic effects.

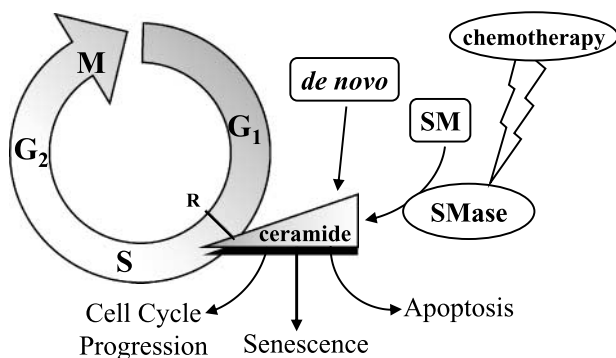


FIGURE 4. Model of ceramide control over the cell cycle; senescence or cell death. During G₁, ceramide levels increase until the G₁-S restriction point, at which time the fate of the cell is determined. The intracellular level of ceramide governs this decision with low concentrations of ceramide leading to a continuation of the cell cycle, moderate levels causing the cell to enter into senescence, and high ceramide levels shunting the cell into apoptosis. Ceramide levels are controlled by the activities of the *de novo* biosynthetic pathway, SMase(s) and other metabolic pathways that use ceramide as a substrate (i.e., sphingomyelin and ganglioside biosynthesis, and ceramide hydrolysis).

Materials and Methods

Cells and Reagents

AsPc1 and Panc1 cells were purchased from the American Type Culture Collection and were cultured in DMEM (Irvine Scientific) supplemented with 10% fetal bovine serum (Hyclone Laboratories) and 1% each of glutamine, sodium pyruvate, nonessential amino acids, and penicillin/streptomycin. The cells were maintained at 37°C with 5% CO₂. Gemcitabine (Gemzar) was purchased from Eli Lilly, powdered sphingomyelin was from Avanti Polar Lipids; imipramine, manumycin A, and myriocin were from Calbiochem/EMD, and 1S,2R-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (MAPP), 3-O-methylsphingomyelin (Me-SM), and DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) were from BioMol. C₁₂-FDG was from Invitrogen, and MTT and all other chemicals were from Sigma-Aldrich. Sphingomyelin micelles were prepared by repeatedly extruding (15 times) a 100 mg/mL suspension of sphingomyelin in 0.9% sterile saline through 1,000 and 100 nm pore size polycarbonate filters at 70°C to 80°C.

Cell Viability

Cells were seeded into 96-well dishes (1,000 cells/well) and allowed to adhere overnight before the addition of drugs. After 4 d (approximately four doublings), the medium was removed and replaced with serum-free DMEM containing 0.5 mg/mL of MTT (25). The cells were incubated a further 2 to 4 h, at which time the medium was removed, the cells were solubilized with DMSO, and the absorbance at 570 nm was measured. The IC₅₀ is the concentration of drug necessary to reduce growth by 50%. The CI was determined by median effect analysis (26, 27) and used the formula, $CI = D_1/Dx_1 + D_2/Dx_2 + (D_1D_2 / Dx_1Dx_2)$, where D is the IC₅₀ of each drug in the combination and Dx is the IC₅₀ of each drug individually, with drug 1 being sphingomyelin and drug 2 being, for instance, gemcitabine. CI is a numerical value indicating synergism (CI < 0.9), additivity (1.1 > CI > 0.9), or antagonism (CI > 1.1). The “fold change” was calculated by dividing the IC₅₀ in the absence of sphingomyelin by the IC₅₀ in the presence of sphingomyelin (6).

Senescence Assay

Senescence-associated β-galactosidase activity was determined using C₁₂-FDG (lipophilic fluorescein-di-β-D-galactopyranoside) that is nonfluorescent as the digalactoside and monogalactoside and spontaneously incorporates into lipid bilayers. β-Galactosidase activity cleaves the galactose moiety from the molecule, leaving the fluorescent molecule associated with the membrane. Cells in 24-well dishes at 10⁴ cells per well were treated with drugs in the presence of 5 μmol/L of C₁₂-FDG for 4 d, detached with 2 mmol/L of EDTA, and analyzed by flow cytometry (FACSCalibur; Becton Dickinson) in the fluorescein channel. Arbitrary units (median channel fluorescence) are reported. Cells were costained with propidium iodide (5 μg/mL) to differentiate live cells from dead cells.

Cell Cycle Analysis

Cells were seeded at 0.5 × 10⁵ to 1.0 × 10⁵ per T175 flask and allowed to adhere before the media was replaced with medium containing 100 nmol/L of gemcitabine and/or 0.2 mg/mL of sphingomyelin. After 4 d, the cells were harvested and

brought to 70% ethanol, refrigerated overnight, washed twice with PBS, and incubated for 1 h at 37°C in propidium iodide solution (PBS, 50 μg/mL propidium iodide, 0.1% Triton X-100, 20 μg/mL RNase) before analysis by flow cytometry. Marker regions for sub-G₁, G₁-G₀, S, and G₂-M were set manually and the percentage of cells in each region relative to the total in all regions is reported.

Preparation of Cell Lysates

Cells (2.5 × 10⁵ to 5 × 10⁵) were seeded into 175 cm² flasks and incubated overnight to allow attachment. The media and nonadherent cells were removed and replaced with gemcitabine and/or sphingomyelin and incubated for 96 h (approximately four doublings) with drug(s). Cells (both adherent and nonadherent cells) were harvested by scraping and collected in conical tubes. An aliquot (~5%) was removed for quantification of apoptosis induction. The cells were washed twice with PBS [NaPO₄ (pH 7.2) and 0.15 mol/L NaCl] and transferred to 1.5 mL Eppendorf tubes. The cell pellets were resuspended in 100 μL of deionized water, 10% SDS was added to give a final concentration of 1%, and the cells were lysed by drawing each sample through an insulin syringe five times. The protein concentration of the samples was estimated from the equation: protein (mg/mL) = (1.56A₂₈₀) - (0.764A₂₆₀). This equation is from the nomograph prepared by E. Adams (distributed by the California Corporation for Biochemical Research, Los Angeles, CA), which was based on the extinction coefficient for enolase and nucleic acid (28). The lysates were stored at -80°C until use.

Western Blotting

Samples (100 μg total protein) were mixed with sample buffer [final concentration of 2% SDS (w/v), 60 mmol/L Tris-Cl (pH 8.0), 10% glycerol, 4% β-mercaptoethanol, and 0.02% bromophenol blue], passed through an insulin syringe, heated for 5 min at 90°C and separated on 4% to 20% SDS gradient gels (Life Therapeutics) using the supplied Tris-HEPES-SDS running buffer. Proteins were transferred to polyvinylidene fluoride microporous membranes (Millipore) for 90 min at 40 V. Blots were blocked overnight at 4°C with 1% casein + 0.05% Tween 20. The blots were probed with antibodies for actin (Sigma) and p21 (Cell Signaling Technologies) for 1 h at room temperature. The Phototope-HRP Western blot detection system (Cell Signaling Technologies) was used to visualize protein bands.

Statistical Analysis

Student's *t* test was used to assess relatedness, with $P < 0.05$ indicating statistically significant differences. CI was determined by median effect analysis (26, 27).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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