Original Paper

Sphingosine Suppresses Mesothelioma Cell Proliferation by Inhibiting PKC- δ and Inducing Cell Cycle Arrest at the G₀/G₁ Phase

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Key Words

Sphingosine • Protein kinase C- δ • Mesothelioma cell • Proliferation • Suppression

Abstract

Background/Aims: Sphingosine regulates cellular differentiation, cell growth, and apoptosis. The present study aimed at understanding sphingosine-regulated mesothelioma cell proliferation. **Methods:** Human malignant mesothelioma cells such as NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cells were cultured. The siRNA to silence the protein kinase C (PKC)- δ -targeted gene was constructed and transfected into cells. MTT assay, cell cycle analysis using a flow cytometry, and cell-free PKC- δ assay were carried out. **Results:** For all the cell types sphingosine inhibited cell growth in a concentration (1-100 μ M)-dependent manner. The sphingosine effect was not prevented by rottlerin, an inhibitor of protein kinase C- δ (PKC- δ); conversely, rottlerin further enhanced the sphingosine effect or rottlerin suppressed mesothelioma cell growth without sphingosine. In the cell-free PKC assay, sphingosine attenuated PKC- δ activity. Knocking-down PKC- δ induced cell cycle arrest at the G₀/G₁ phase and inhibited cell growth. **Conclusion:** The results of the present study show that sphingosine suppressed mesothelioma cell proliferation by inhibiting PKC- δ , to induce cell cycle arrest at the G₀/G₁ phase.

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Introduction

Sphingolipids include ceramide, ceramide 1-phosphate, sphingosine, and sphingosine 1-phosphate (S1P). Sphingosine is produced from ceramidase-catalyzed cleavage of fatty acids from ceramide. Evidence has pointed to the role of sphingolipid in the regulation of cellular differentiation, cell growth, and apoptosis. We have earlier found that sphingosine induces apoptosis in hippocampal neurons and astrocytes by activating caspase-3/-9 via a sphingosine-dependent protein kinase (SDK)/14-3-3 protein/Bax/cytochrome c pathway [1]. SDK is produced through proteolytic processing of protein kinase C- δ (PKC- δ) and activated by binding sphingosine [2]. SDK specifically phosphorylates 14-3-3 protein [3, 4], thereby dissociating Bax from a complex with 14-3-3 protein, to induce a mitochondria-mediated apoptosis. Sphingosine, alternatively, induces apoptosis in rhabdomyosarcoma cells by activating caspase-3/-9 in a Bax-dependent manner [5] or in mouse BALB/c 3T3 clone A31 cells in an SDK-dependent manner [2]. In our recent study, sphingosine induced apoptosis in well differentiated MKN-28 human gastric cancer cells by increasing SDK production from PKC- δ , to phosphorylate 14-3-3 protein, thereby causing disruption of mitochondrial membrane potentials and activating caspase-9 followed by the effector caspase-3. Sphingosine, thus, might be a target for development of anti-tumor drugs.

Malignant mesothelioma is an aggressive tumor arising from previous asbestos exposure. A great deal of challenge has been attempted, yet disappointingly, malignant mesothelioma is considerably resistant to conventional anticancer therapies and no beneficial effect is expected. Then, we were prompted to assess the effect of sphingosine on proliferation of malignant mesothelioma cells. We show here that sphingosine suppresses mesothelioma cell proliferation by inhibiting PKC- δ , to induce cell cycle arrest at the G₀/G₁.

Materials and Methods

Cell culture

NCI-H28, NCI-H2052, NCI-H2452, and MSTO-211H cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.003% (w/v) L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Assay of cell viability

Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [1]. MTT-reactive cells were quantified at an absorbance of 570 nm using a micro-plate reader (SPECTRAmax PLUS384, Molecular Devices, Sunnyvale, CA, USA).

Cell cycle analysis

Cells were harvested by a trypsinization, fixed with 70% (v/v) ethanol at 4 °C overnight. Fixed cells were incubated in phosphate-buffered saline containing 1.5 µg/ml RNase A for 1 h at 37 °C, followed by staining with 5 µl of propidium iodide (PI) for 20 min on ice. Then, cells were collected on a nylon mesh filter (pore size, 40 µm), and cell cycles were assayed using a flowcytometer (FACSCalibur, Becton Dickinson, USA) at an excitation of 488 nm and an emission of 585 nm, and analyzed using FlowJo software (TreeStar, San Carlos, CA, USA).

Cell-free PKC- δ assay

PKC activity in the cell-free systems was quantified by the method as previously described [6]. Briefly, synthetic PKC substrate peptide (10 µM) was reacted with PKC- δ in a Ca²⁺-free medium containing 20 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, and 10 µM ATP with and without sphingosine in the absence of phosphatidylserine and diacylglycerol at 30 °C for 5 min. After loading on a reversed phase high performance liquid chromatography (HPLC)(LC-10ATvp, Shimadzu Co., Kyoto, Japan), a substrate peptide peak and a new

Fig. 1. Sphingosine-induced suppression of cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with sphingosine at concentrations as indicated for 24–48 h, and then, MTT assay was carried out. In the graphs, each point represents the mean ($\pm$ SEM) percentage of basal cell viabilities (MTT intensities before treatment with sphingosine) ($n=4$ independent experiments).

product peak were detected at an absorbance of 214 nm. Areas for non-phosphorylated and phosphorylated PKC substrate peptide were measured (total area corresponds to concentration of PKC substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/1 min) was used as an index of PKC- δ activity.

Construction and transfection of siRNA

The siRNA to silence PKC- δ -targeted gene (PKC- δ siRNA) and the negative control siRNA (NC siRNA) were obtained from BONAC (Fukuoka, Japan). The PKC- δ siRNA and the NC siRNA were reverse-transfected into cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Western blotting

Cells were lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a TGX gel (BioRad, Hercules, CA, USA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently incubated with an anti-PKC- δ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or an anti- β -actin antibody (Sigma, St Louis, MO, USA). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ, USA) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce, Rockford, IL, USA).

Statistical analysis

Statistical analysis was carried out using Fisher's Protected Least Significant Difference (PLSD) test, unpaired *t*-test, and Dunnett's test.

Results

Sphingosine suppresses mesothelioma cell proliferation

For all the mesothelioma cells examined here treatment with sphingosine for 24–48 h decreased the number of viable cells in a concentration (1–100 μ M)-dependent manner,

Fig. 2. The effect of S1P on cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with S1P at concentrations as indicated for 24-48 h, and then, MTT assay was carried out. In the graphs, each point represents the mean ($\pm$ SEM) percentage of basal cell viabilities (MTT intensities before treatment with S1P) (n=4 independent experiments).

Fig. 3. The effect of sphingosine on SDK production. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with sphingosine (100 μM) for 0-6 h, and Western blotting was carried out using an anti-PKC-δ antibody. Note that no increase in the SDK production (arrows) was found with sphingosine treatment and that similar results were obtained with 4 independent experiments.

without difference in the extent among the cell types (Fig. 1A, B, C, D). In the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, no significant increase in TUNEL-positive cells was obtained with sphingosine for all the cell types (data not shown). This suggests that sphingosine does not induce mesothelioma cell apoptosis but suppresses mesothelioma cell proliferation.

Sphingosine is phosphorylated by sphingosine kinase 1 to produce S1P. Treatment with S1P for 24-48 h had little effect on the number of viable cells at concentrations ranging from 1 to 30 μM for all the cell types (Fig. 2A, B, C, D). This indicates that sphingosine by itself, but not S1P produced from sphingosine, has the potential to suppress mesothelioma cell proliferation.

Fig. 4. The effect of rottlerin on sphingosine-induced suppression of cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with sphingosine at concentrations as indicated in the presence and absence of rottlerin (10 μ M) for 24 h, and then, MTT assay was carried out. In the graphs, each point represents the mean ($\pm$ SEM) percentage of basal cell viabilities (MTT intensities before treatment with sphingosine in the absence of rottlerin)(n=4 independent experiments).

Fig. 5. Rottlerin-induced suppression of cell viability. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were treated with rottlerin alone at concentrations as indicated for 24-48 h, and then, MTT assay was carried out. In the graphs, each point represents the mean ($\pm$ SEM) percentage of basal cell viabilities (MTT intensities before treatment with rottlerin) (n=4 independent experiments).

In our earlier study, sphingosine induced apoptosis in hippocampal neurons and astrocytes via a PKC- δ /SDK pathway [1]. We subsequently examined whether the sphingosine effect is due to PKC- δ /SDK activation. Sphingosine did not increase SDK production in all the cell types (Fig. 3A, B, C, D), ruling out the participation of SDK in the sphingosine effect here. Sphingosine-induced decrease in the number of viable cells was not inhibited by rottlerin (10 μ M), an inhibitor of PKC- δ ; conversely, further decrease in the number of viable cells was found in the presence of rottlerin ($P < 0.001$ as compared with the effect of sphingosine in the absence of rottlerin for all the cell types, Fisher's PLSD test) (Fig. 4A, B, C, D). Moreover, treatment with rottlerin alone for 24-48 h decreased the number of viable cells in a concentration (0.1-10 μ M)-dependent manner for all the cell types (Fig. 5A, B, C,

Fig. 6. Sphingosine-induced PKC- δ inhibition. In the cell-free systems, PKC- δ activity was assayed in the presence and absence of sphingosine (100 μ M). In the graph, each column represents the mean ($\pm$ SEM) PKC- δ activity (pmol/min)(n=4). *P* value, unpaired *t*-test.

Fig. 7. The effect of PKC- δ knock-down on cell viability. MTT assay was carried out in NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) transfected with the NC siRNA (NC) or the PKC- δ siRNA (PKC- δ KD). Note that expression of PKC- δ is drastically decreased in all the cell types transfected with the PKC- δ siRNA in the Western blot analysis. In the graphs, each point represents the mean ($\pm$ SEM) percentage of basal cell viabilities (MTT intensities for cells transfected with NC siRNA at 48 h after transfection)(n=4 independent experiments). *P* values, Fisher's PLSD test.

D). Taken together, these results suggest that PKC- δ promotes proliferation of mesothelioma cells. Then, we postulated that sphingosine might suppress proliferation of mesothelioma cells by inhibiting PKC- δ .

Mesothelioma cell proliferation is suppressed by inhibiting PKC- δ

To obtain evidence for sphingosine-induced PKC- δ inhibition, we carried out cell-free PKC assay. PKC- δ activity in the absence of sphingosine was 2.26 ± 0.32 pmol/min, but the activity was significantly attenuated in the presence of sphingosine (0.76 ± 0.06 pmol/min) (Fig. 6). This confirms that sphingosine inhibits PKC- δ .

For cells transfected with the PKC- δ siRNA, expression of PKC- δ protein was clearly reduced than the expression for cells transfected with NC siRNA (Fig. 7A, B, C, D), confirming PKC- δ knock-down. Spontaneous mesothelioma cell growth was significantly inhibited

Fig. 8. The effect of PKC- δ knock-down on cell cycling. NCI-H28 (A), NCI-H2052 (B), NCI-H2452 (C), and MSTO-211H cells (D) were transfected with the NC siRNA (NC) or the PKC- δ siRNA (PKC- δ KD), and 72 h later cell cycle analysis was carried out. In the graphs, each column represents the mean ($\pm$ SEM) percentage for phases of cell cycling (n=4 independent experiments). P values, Dunnett's test.

by knocking-down PKC- δ , with the order of the potential: NCI-H2052 cells=MSTO-211H cells>NCI-H2452 cells>>NCI-H28 cells (Fig. 7A, B, C, D). This indicates that PKC- δ promotes mesothelioma cell proliferation.

Sphingosine suppresses mesothelioma cell proliferation by inducing cell cycle arrest at the G₀/G₁ phase

In the cell cycle analysis, knocking-down PKC- δ significantly increased the population of cells at the G₀/G₁ phase of cell cycling, but it otherwise decreased the population at the G₂/M phase for all the cell types (Fig. 8). This implies that PKC- δ accelerates cell cycling for mesothelioma cells, i.e., inhibiting PKC- δ causes cell cycle arrest at the G₀/G₁ phase.

Discussion

The results of the present study demonstrate that sphingosine suppresses mesothelioma cell proliferation. Sphingosine is recognized to bind to and activate SDK, that is produced through proteolytic processing of PKC- δ [2]. Sphingosine induces apoptosis in hippocampal neurons and astrocytes by activating SDK, to phosphorylate 14-3-3 protein, thereby dissociating Bax and disrupting mitochondrial membrane potentials, and then leading to activation of caspase-9 and the effector caspase-3 [1]. In the present study, sphingosine did not increase SDK production, which would exclude the possibility for the implication of SDK in sphingosine-induced suppression of mesothelioma cell proliferation.

Of PKC isozymes cloned PKC- δ as well as the other novel PKCs including PKC- ϵ , - η , - θ , and - μ , is activated by signaling cascades linked to phospholipase A₂ in a Ca²⁺-independent manner [7, 8]. PKC- δ is activated by translocation towards the membrane surface [9]. Sphingosine inhibits PKC activation induced by diacylglycerol, phorbol dibutyrate, calcium, unsaturated fatty acids or other lipids [10, 11]. PKC binds to membranes through interactions with diacylglycerol and negatively charged phosphatidylserine. Sphingosine may be localized

in regions of acidic lipids, thereby inhibiting PKC binding to membranes and activity [12-15]. Sphingosine, alternatively, inhibits the enzyme phosphatidic acid phosphohydrolase, that generates diacylglycerol production from phosphatidic acid produced by phospholipase D-catalyzed hydrolysis of phosphatidylcholine [16-18]. In the cell-free PKC assay, sphingosine attenuated PKC- δ activity. This indicates that sphingosine inhibits PKC- δ through its direct binding. Notably, rottlerin, an inhibitor of PKC- δ , further enhanced sphingosine-induced suppression of mesothelioma cell growth or rottlerin alone without sphingosine inhibited mesothelioma cell growth. Moreover, knocking-down PKC- δ significantly suppressed mesothelioma cell growth. It is indicated from these results that sphingosine suppresses mesothelioma cell proliferation by inhibiting PKC- δ . In the cell cycle analysis, knocking-down PKC- δ induced cell cycle arrest at the G₀/1 phase for all the cell types used here. Overall, these results lead to a conclusion that sphingosine suppresses mesothelioma cell proliferation by inhibiting PKC- δ , to induce cell cycle arrest at the G₀/1 phase.

Lines of evidence have pointed to the contrasting roles of PKC- δ in cell survival and cell death [19]. The pro- and anti-apoptotic function of PKC- δ not only depends on the cell type but also on the stimulus. PKC- δ promotes survival of a variety of cancer cells such as non-small cell lung cancer, breast cancer, pancreatic cancer, liver cancer, and chronic lymphocytic leukemia cells. PKC- δ protects glioma cells from the apoptosis induced by TRAIL, and phosphorylation of PKC- δ on Tyr155 and its cleavage are essential for the anti-apoptotic effect of PKC- δ [20]. Phosphorylation of PKC- δ at Tyr332 is also required for protection against TRAIL-induced apoptosis [21]. Overexpression of PKC- δ increases cell proliferation, anchorage-independent growth, and resistance to apoptotic stimuli by elevating cyclin D1 level and hyperphosphorylating Rb in murine mammary NMuMG cells [22]. Conversely, PKC- δ antisense oligonucleotide and dominant-negative PKC- δ decrease survival of breast cancer MCF-7 and MDA-MB-231 cells [23]. As found with the present study, rottlerin enhances apoptosis in non-small cell lung cancer cells [24]. Rottlerin blocks DNA damage-induced apoptosis, but otherwise it potentiates receptor-induced apoptosis [25].

PKC- δ promotes cell survival via several well-known pro-survival pathways, that include NF- κ B, Akt, and ERK. PKC- δ prevents apoptosis in colon cancer cells by inducing inhibitor of apoptosis protein-2 and FLICE-like inhibitory protein via NF- κ B [26, 27]. PKC- δ suppresses autophagy in breast cancer cells via NF- κ B [28]. Tumor necrosis factor (TNF) is recognized to induce translocation of PKC- δ to the nucleus, where it bound to the NF- κ B RelA subunit and induced transactivation of p65/RelA [29]. PKC- δ protects MCF-7 breast cancer cells against TNF-related apoptosis-inducing ligand-mediated apoptosis [30]. Phosphoinositide-dependent kinase-1 may also participate in PKC- δ survival signaling in cells containing an activated p21Ras protein [31]. Activation of Akt and ERK, alternatively, is responsible for PKC- δ -mediated increase in anchorage-independent growth and resistance of pancreatic ductal cancer cells to apoptotic stimuli [32]. PKC- δ -induced cell proliferation in murine mammary cells is associated with activation of ERK/MAPK [22]. In contrast, PKC- δ -induced suppression of ERK1/2 is associated with the survival of MDA-MB-231 cells [33]. PKC- δ attenuates apoptosis by inducing phosphorylation and proteasomal degradation of the proapoptotic protein Bim via the MEK/MAPK pathway in immortalized and malignant keratinocytes [34]. Syk, a tyrosine kinase, promotes survival of B-cell chronic lymphocytic leukemia cells by stabilizing Mcl-1 in a PKC- δ -dependent manner [35]. PKC- δ activated by Syk phosphorylates and inhibits glycogen synthase kinase-3, causing stabilization of Mcl-1 and inhibition of apoptosis. It is presently unknown what signaling pathways underlie sphingosine/PKC- δ -dependent inhibition of proliferation in mesothelioma cells. To address this question, we are carrying out further experiments.

In conclusion, the results of the present study show that sphingosine inhibits PKC- δ , thereby inducing cell cycle arrest at the G₀/1 phase, and then leading to suppression of mesothelioma cell proliferation. This may represent further insight into the sphingosine signaling pathway relevant to cell proliferation and death.

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