Evaluation of chemotherapeutic and cancer-protective properties of sphingosine and C_2-ceramide in a human breast stem cell derived carcinogenesis model

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Received May 8, 2018; Accepted August 30, 2018

DOI: 10.3892/ijo.2018.4641

Abstract. The overall goal of the present study was to evaluate the chemotherapeutic and cancer-protective properties of D-*erythro*-sphingosine (sphingosine) and C_2-ceramide using a human breast epithelial cell (HBEC) culture system, which represents multiple-stages of breast carcinogenesis. The HBEC model includes Type I HBECs (normal stem), Type II HBECs (normal differentiated) and transformed cells (immortal/non-tumorigenic cells and tumorigenic cells, which are transformed from the same parental normal stem cells). The results of the present study indicate that sphingosine preferentially inhibits proliferation and causes death of normal stem cells (Type I), tumorigenic cells, and MCF7 breast cancer cells, but not normal differentiated cells (Type II). In contrast to the selective anti-proliferative effects of sphingosine, C_2-ceramide inhibits proliferation of normal differentiated cells as well as normal stem cells, tumorigenic cells, and MCF7 cancer cells with similar potency. Both sphingosine and C_2-ceramide induce apoptosis in tumorigenic cells. Among the sphingosine stereoisomers (D-*erythro*, D-*threo*, L-*erythro,* and L-*threo*) and sphinganine that were tested, L-*erythro*-sphingosine most potently inhibits proliferation of tumorigenic cells. The inhibition of breast tumorigenic/cancer cell proliferation by sphingosine was accompanied by inhibition of telomerase activity. Sphingosine at non-cytotoxic concentrations, but not C_2-ceramide, induces differentiation of normal stem cells (Type I), thereby reducing the number of stem cells that are more susceptible to neoplastic transformation. To the best of our knowledge, the present study demonstrates one of the first results that sphingosine can be a potential chemotherapeutic and cancer-protective agent, whereas C_2-ceramide is not an ideal chemotherapeutic and cancer-protective agent due to its anti-proliferative effects on Type II HBECs and its inability to induce the differentiation of Type I to Type II HBECs.

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Key words: sphingosine, sphingosine stereoisomers, sphinganine, ceramide, proliferation, apoptosis, differentiation, telomerase, breast cancer, stem cells

Introduction

Breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer associated-mortality in women in the United States (1). The majority of breast cancer types are adenocarcinomas (invasive ductal carcinoma) originating from epithelial cells (2). Terminal end buds, which contain proliferating mammary epithelial stem cells, appear to be the target of mammary neoplastic transformation (3,4). The development of chemotherapeutic agents without side effects and drug resistance has been an active area of cancer research.

Sphingolipids are present in all eukaryotic cell membranes, specific prokaryotes, and in certain foods (5,6). Anti-tumorigenic *in vivo* effects of complex sphingolipids could be the result of the conversion of complex sphingolipids to sphingolipid metabolites including sphingosine, sphinganine and ceramide. Ceramides are composed of sphingosine and a fatty acid with a variable chain length. These sphingolipid metabolites function as second messengers in signal transduction pathways to regulate cell proliferation, differentiation, apoptosis, and cell migration (6,7). Sphingosine is a metabolic precursor to sphingosine-1-phosphate (S1P), however sphingosine and S1P exhibit opposing effects (e.g., sphingosine is anti-proliferative and pro-apoptotic; S1P is growth-stimulatory and anti-apoptotic effects) (6-8). It is suggested that the balance between sphingosine and S1P can form a rheostat model: When this balance moves toward sphingosine it triggers death of the cancer cell, whereas increased S1P levels lead to cancer cell survival (8). Most studies have examined effects of ceramides and S1P, while fewer have been performed on elucidating the action of sphingosine, its stereoisomers, and sphinganine.

Previously the authors have developed a stem cell-derived breast carcinogenesis model that encompasses Type I and Type II normal human breast epithelial cells (HBECs) and transformed cells, which represent multiple stages of breast carcinogenesis. Type I HBECs display stem cell characteristics and have been characterized by: The expression of a stem cell marker octamer-binding transcription factor 4 (9), estrogen receptor α (10), and luminal epithelial markers (11,12); a deficiency in gap-junction associated intercellular communication (11,13); the ability to display anchorage-independent growth (13); the ability to differentiate into Type II (normal differentiated) HBECs (11,13); reduced expression of maspin (14); and the ability to form budding/ductal organoids on Matrigel in conjunction with Type II HBECs (13). Furthermore, Type I HBECs have been sequentially transformed into immortal/non-tumorigenic cells (M13SV1), weakly tumorigenic cells (M13SV1R2) and highly tumorigenic cells (M13SV1R2N1) by oncogenic treatments, the SV40 large T-antigen (SV40-T), X-rays, and the receptor tyrosine-protein kinase erbB-2/neu oncogene (11,15). Type I HBECs are more susceptible to the oncogenic treatments than Type II HBECs. In contrast, Type II HBECs rarely become immortal following transfection with SV40-T (10,11,13,16). Type II HBECs demonstrate basal epithelial phenotypes and do not express the estrogen receptor α. This suggests that Type I HBECs appear to be the major target cells for breast carcinogenesis.

The unique HBEC model system described above has enabled the authors to evaluate chemotherapeutic and cancer-protective properties of sphingolipid metabolites. The effects of sphingosine, its stereoisomers, sphinganine, and C_2-ceramide (N-acetyl-D-*erythro*-sphingosine) have been evaluated on Type I (normal stem) HBECs, Type II (normal differentiated) HBECs, and highly tumorigenic cells transformed from the parental normal stem cells. In addition, the effects of sphingosine and C_2-ceramide were tested on MCF7 breast cancer cells. The following criteria were applied to define chemotherapeutic and cancer-protective agents: i) A chemotherapeutic agent is expected to preferentially inhibit the proliferation and induce apoptosis in tumorigenic cells and normal stem cells (Type I), while it has no or little effect on normal differentiated cells (Type II); ii) A potential cancer-protective agent is expected to induce the differentiation of normal stem cells (Type I) to normal differentiated cells (Type II), thereby decreasing the number of stem cells that are more susceptible to neoplastic transformation.

Materials and methods

Sphingolipids. D-*erythro*-sphingosine (hereinafter referred to as sphingosine) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Sphingosine stereoisomers (D-*threo*, L-*threo,* and L-*erythro*), sphinganine (D-*erythro*-dihydro-sphingosine), and C_2-ceramide were obtained from Matreya LLC (State College, PA, USA). These sphingolipid metabolites were dissolved and prepared at working concentrations as described previously (14,17). During experimental periods, sphingosine and sphingosine stereoisomers were delivered to cells in 0.1% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA).

Development of normal HBECs from reduction mammoplasty tissues and derivation of in vitro neoplastically transformed cells. Normal HBECs were developed as described previously (11,14). *In vitro* neoplastically transformed HBEC lines (M13SV1, M13SV1R2, and M13SV1R2N1) were sequentially derived from Type I HBECs (11,13,15,18). In the present study, Type I and Type II HBECs and the human breast transformed highly tumorigenic cells (M13SV1R2N1) were tested. The transformed highly tumorigenic cells (M13SV1R2N1, hereinafter referred to as tumorigenic cells) examined in the present study were authenticated by short tandem repeat (STR) DNA profiling (Genetica DNA Laboratories, Cincinnati, OH, USA). The STR DNA-profile of the tumorigenic cells is unique among the known 3,274 cell lines reposited in American Type Culture Collection (ATCC, Manassas, VA, USA), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Japanese Collection of Research Bioresources, or RIKEN and does not match any of these known repository cell lines, indicating no cross-contamination or misidentification of the cells. The STR DNA-profiling results for tumorigenic cells are presented in brackets for each STR locus: D3S1358 *[14]*, TH01 *[5]*, D21S11 *[28]*, D18S51 *[12,21]*, Penta E *[11]*, D5S818 *[11,12]*, D13S317 *[9]*, D7S820 *[8,9]*, D16S539 *[12,15]*, CSF1PO *[12,13]*, Penta D *[9,13]*, vWA *[14,17]*, D8S1179 *[12,15]*, TPOX *[11]*, FGA *[21]*, Amelogenin/Gender marker *[X]*.

Cell culture and assessment of cell proliferation. Type I and Type II HBECs and tumorigenic cells (M13SV1R2N1) were cultured in the MSU-1 medium with 'supplements (S)' (called 'MSU-1+S medium' hereafter) as described previously (14). The composition of MSU-1 media (11) is equivalent to the 1:1 (vol:vol) mixture of DMEM with low glucose and keratinocyte-serum free media (both from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The 'supplements' (11) are 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 4 μg/ml transferrin (all from Sigma-Aldrich; Merck KGaA), 0.5 ng/ml epidermal growth factor (EGF; Invitrogen; Thermo Fisher Scientific, Inc.), 10^{-8} M 17-β-estradiol (Sigma-Aldrich, Merck KGaA), and 50 μg/ml gentamicin (Invitrogen; Thermo Fisher Scientific, Inc.). MCF7 breast cancer cells, obtained from the ATCC, were cultured using the same procedures as for the breast tumorigenic cells (M13SV1R2N1). Total nucleic acid concentrations representing cell numbers were determined as described previously (14).

Analysis of DNA fragmentation by agarose gel electrophoresis. Cells were trypsinized and centrifuged at 379 x g at 4˚C for 10 min. Pellets were resuspended in 200 μl PBS and DNA was extracted with 400 μl phenol/chloroform, and then was centrifuged at max speed (13,000 x g) at 4˚C for 5 min. Following incubation with 0.2 mg/ml RNase A at 37˚C for 1 h, the extraction was repeated with 400 μl phenol/chloroform to inactivate RNase A. A total of 20 μl of 3 M NaOAc and 500 μl of 100% ethanol were added to the solution prior to storing at -20˚C overnight. Following centrifugation at max speed (13,000 x g) at 4˚C for 30 min, the DNA pellets were washed with 70% ethanol and dried *en vacuo*. Finally, the pellets were dissolved in Tris-EDTA (pH 8) and the 5 μg DNA was separated on a 2% agarose gel at 100 V for 1 h and the gel was stained with ethidium bromide.

Flow cytometry analysis of apoptotic cells. Cells were collected and resuspended in DNA staining reagent [0.1 mM EDTA (pH 7.4), 0.1% of Triton X-100, 0.05 mg/ml RNase A (50 U/mg) and 50 μg/ml propidium iodide (Thermo Fisher Scientific, Inc.) in PBS at pH 7.4], and then were processed as described previously (14). Cells were analyzed via Fluorescence-activated cell sorting (FACS) Vantage cytometer (BD Biosciences, San Jose, CA, USA). The percentage of cells in each phase of the cell cycle and apoptotic cells was calculated using the FCS Express 2.0 and Win cycle (MultiCycle AV) programs (Phoenix Flow Systems, San Diego, CA, USA). Apoptotic cells (A_0 peaks) with reduced DNA content were determined as the percentage of cells in the hypo-diploid sub G_0/G_1 area to the left of G_0/G_1 diploid peak.

Assessment of Type I HBEC differentiation. Differentiation of Type I to Type II HBEC experiments were conducted as described previously (14). At the end of each differentiation culture period, Type I and Type II colonies were visually identified using a light microscope by placing the cell culture 60 mm dish into the dish holder of the microscope and sliding the stage in a controlled manner. Then, the Type I and Type II colonies of each differentiation culture dish were counted, respectively. The identity of the sphingolipid and cholera toxin (Sigma-Aldrich; Merck KGaA) treatments was unknown (blind) during counting to ensure objectivity.

Polymerase chain reaction (PCR)-based telomerase assay. Cells were harvested by trypsinization. Following cell counting, the cells were centrifuged at 78 x g at 4˚C for 6 min to remove trypsin solution. Cell pellets were washed with 10 ml PBS and then centrifuged at 113 x g at 4˚C for 8 min to remove the PBS. Cells were then suspended at $1x10^6$ cells/ml in PBS and aliquoted into Eppendorf tubes. After cells were centrifuged at 176 x g at 4˚C for 8 min and the PBS was carefully removed, the cell pellets were stored at -80˚C. Telomerase activities were measured using the TRAPeze™ Telomerase Detection kit (cat. no. S7700; EMD Millipore, Billerica, MA, USA) and the procedures were performed according to the company's protocol. The cell pellets were thawed and resuspended in 200 μl of 1X CHAPS lysis buffer (EMD Millipore)/10^6 cells and left on ice for 30 min. Samples were centrifuged at 12,000 x g for 20 min at 4˚C. The TRAPeze kit includes a 36 base pair primer to serve as an internal standard for amplification, therefore providing a positive control for accurate quantitation of telomerase activity. Each analysis included a negative control (CHAPS-lysis buffer without sample), heat-inactivated control (sample incubated at 85˚C for 10 min prior to the assay), and a positive cell line control (MCF7 breast cancer cells: See the section of Methods-Cell culture). The products (25 μl) of the telomerase repeat amplification protocol (TRAP) assay were resolved via a non-denaturing 12% PAGE in a buffer containing 54 mM Tris-HCL (pH 8.0), 54 mM boric acid, and 1.2 mM EDTA. The gel was stained with SYBR-Green for ~30 min at room temperature (Molecular Probes, Inc; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and visualized using a 302 nm UV transilluminator. The density of DNA bands was analyzed/quantified by AlphaImager™ 3300 (Alpha Innotech Corporation, San Leandro, CA, USA) and Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA).

Statistical analysis. Statistical analyses were carried out using Sigma plot version 12.0 (Systat Software, Inc., San Jose, CA, USA) or R (program version 3.0.2 for Windows www.r-project.org). Data of cell growth at different concentrations of sphingolipids and multiple culture periods were analyzed by two-way factorial analysis of variance (ANOVA). A one-way ANOVA was applied for the cell growth data at different concentrations of sphingolipids for a single culture period. Following the application of two-way ANOVA or one-way ANOVA, the Holm-Sidak method was applied for multiple comparisons. Differences between the control and sphingosine groups at a single concentration for: Apoptotic cells at pre-G_0/G_0 regions and telomerase activity results were analyzed by Mann-Whitney rank sum test and t-test, respectively. $P<0.05$ was considered to indicate a statistically significant difference, unless otherwise specified. Differentiation of Type I to Type II HBEC results was analyzed by Chi-Square test. For the differentiation data with multiple (>two) groups and with $P<0.05$, post-hoc tests were done by conducting multiple pairwise Chi-Square tests with Bonferroni corrections. P-value cutoff used to identify statistical significance was adjusted for each set of pairwise comparisons as part of the Bonferroni correction (Adjusted P-value =0.05/number of pairwise comparisons). Data were considered statistically significant if the P-value of each pairwise comparison was below the Bonferroni correction adjusted P-value.

Results

Sphingosine selectively inhibits proliferation and causes death of normal stem cells (Type I) and tumorigenic/cancer cells, but not normal differentiated cells (Type II). In normal stem cells (Type I HBECs: Fig. 1A), control cultures grew slowly over the culture period with the total concentration of nucleic acid increasing by ~70% in 5 days. Sphingosine at 2 and 5 μM did not affect cell proliferation; however, sphingosine at 8 μM significantly reduced the nucleic acid concentration to ~60% of the corresponding control cultures at day 1 ($P<0.05$) and floating dead cells were visible in the medium. Thereafter, for cells treated with 8 μM sphingosine, the total nucleic acid concentration remained the same through day 5 suggesting that sphingosine blocked cell proliferation.

In normal differentiated cells (Type II HBECs: Fig. 1B), the control culture cell number tripled in 2 days and remained in log-phase at 5 days of culture. Cell proliferation was not affected by sphingosine at concentrations as high as 8 μM, which was the highest concentration tested.

In breast tumorigenic cells (M13SV1R2N1) and MCF7 breast cancer cells, sphingosine caused concentration and time-dependent decreases in total nucleic acid concentration (Fig. 1C and D). Sphingosine at 5 to 10 μM significantly inhibited the proliferation and caused death of breast tumorigenic cells and MCF7 cells ($P<0.05$). Sphingosine at 5 μM reduced total nucleic acid content to ~50% compared with the control and to ~80% of control at day 5 in breast tumorigenic cells (Fig. 1C) and MCF7 cells (Fig. 1D), respectively.

C_2-ceramide inhibits proliferation and causes cell death in normal stem cells (Type I), normal differentiated cells (Type II), and tumorigenic/cancer cells. For Type I HBECs (Fig. 1E), C_2-ceramide at 2 and 5 μM significantly

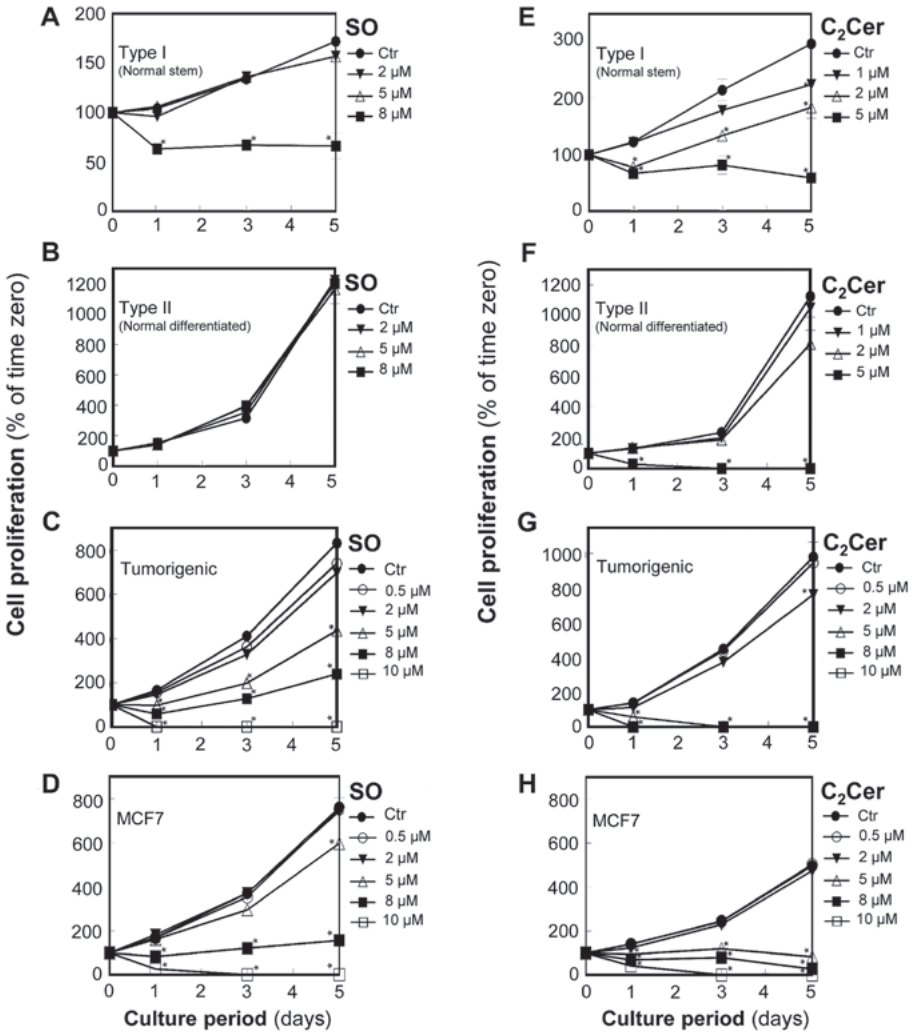

Figure 1. SO selectively inhibits the proliferation of normal stem cells (Type I), tumorigenic cells, and MCF7 cancer cells, but not normal differentiated cells (Type II). In contrast, C_2Cer inhibits the proliferation of all breast cells tested in this study (Type I HBECs, Type II HBECs, tumorigenic cells, and MCF7 cancer cells). Subconfluent cells ($6x10^4$) were cultured in 6-well plates in triplicate and treated with SO (A) Type I HBECs, (B) Type II HBECs, (C) tumorigenic cells, and (D) MCF7 cancer cells and C_2Cer (E) Type I HBECs, (F) Type II HBECs, (G) tumorigenic cells, and (H) MCF7 cancer cells at various concentrations (0.5-10 μM) on day 0 and day 3. Total nucleic acid concentrations were measured as an index of cell number for Type I (normal stem: A and E), Type II (normal differentiated: B and F), tumorigenic (C and G), and MCF7 (D and H) cells. Results are expressed as a percentage of the quantity at zero hours (mean ± standard deviation, n=3). *P<0.05 vs. the Ctr. Where an error bar is not seen, it lies within the dimensions of the symbol. HBECs, human breast epithelial cells; Ctr, control; SO, sphingosine; Cer, ceramide.

inhibited cell proliferation (P<0.05) and caused death within 1 day. C_2-ceramide at 5 μM reduced total nucleic acid content to ~60% of the corresponding control at day 1 and to ~25% of the control by day 5 (Fig. 1E). C_2-ceramide at 8 μM killed all of the cells by day 3. For Type II HBECs (Fig. 1F), C_2-ceramide at 5 μM significantly inhibited cell proliferation (P<0.05) and caused cell death within 1 day.

In breast tumorigenic cells (Fig. 1G) and MCF7 breast cancer cells (Fig. 1H), C_2-ceramide significantly inhibited cell proliferation at 5 μM and above, and caused death. C_2-ceramide was more potent than sphingosine as effects of 5 μM C_2-ceramide were similar to those of 8 to 10 μM sphingosine.

The unnatural stereoisomers of sphingosine more potently inhibit proliferation and cause death of breast tumorigenic cells compared with the natural form of sphingosine in tumorigenic cells. To study the structural requirements for sphingosine to inhibit proliferation, three unnatural stereoisomers, D-*threo*, L-*threo*, and L-*erythro*-sphingosine at 5 μM were examined along with the natural form D-*erythro*-sphingosine at 5 μM (Fig. 2). Since the authors of the present study previously demonstrated that sphinganine is more potent than D-*erythro*-sphingosine in inhibiting proliferation and inducing apoptosis (14), the effects of sphingosine isomers with sphinganine at the same concentration were also compared. All three unnatural stereoisomers of sphingosine were more potent compared with D-*erythro*-sphingosine in inhibiting proliferation and causing death of tumorigenic cells. L-*erythro*-sphingosine was the most potent among the tested stereoisomers and sphinganine. All isomers significantly inhibited cell growth compared with the control (P<0.05).

Sphingosine and C_2-ceramide induce apoptosis in normal stem cells (Type I) and tumorigenic cells. To examine whether sphingosine and C_2-ceramide caused cell death by inducing apoptosis, the presence of apoptotic cells in Type I HBECs and

Table I. SO induces differentiation of Type I HBECs to Type II HBECs.

Culture (days)	Treatments	Total colonies	Type II containing colonies	% of Type II containing colonies (95% CI)
Type I (normal stem) HBEC from woman ID#1				
5	Ctr for SO (0.1% BSA)	392	26	6.6% (-2.1%)
	ChoT 1 ng/ml	371	231	62.3% (-5.0%)[b]
	SO 1 μM	356	49	13.8% (-3.2%)[a]
	SO 2 μM	187	39	20.9% (-5.2%)[b]
	SO 3 μM	32	7	21.9% (-10.9%)[a]
9	Ctr for SO (0.1% BSA)	322	25	7.8% (-2.5%)
	ChoT 1 ng/ml	335	311	92.8% (-3.3%)[b]
	SO 1 μM	350	92	26.3% (-4.3%)[b]
	SO 2 μM	223	55	24.7% (-5.2%)[b]
	SO 3 μM	43	10	23.3% (-10.1%)[a]

Type I HBECs ($5x10^3$) were cultured in 60 mm dishes with grids in triplicate and treated with SO at non-cytotoxic concentrations (1 to 3 μM). ChoT was used as a positive differentiation inducer. Type I and Type II colonies were quantified at day 5 and day 9 following the first treatments. The % differentiation is calculated by (number of Type II HBEC-containing colonies/total number of colonies) x100. Significant differences compared to the corresponding controls are indicated. [a]P<0.01; [b]P<$5x10^{-5}$ by Chi-square test with Bonferroni corrections. The lower bounds of Wilson score 95% CI are demonstrated in parentheses. CI, confidence intervals; ChoT, Cholera toxin; SO, sphingosine; HBECs, human breast epithelial cells; BSA, bovine serum albumin.

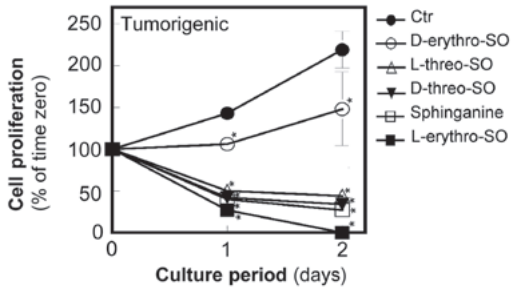

Figure 2. The unnatural stereoisomers of sphingosine more potently inhibit proliferation and cause the death of breast tumorigenic cells compared with the natural form D-*erythro* SO. Subconfluent cells ($6x10^4$) were cultured in 6-well plates in triplicate and treated with 5 μM SO stereoisomers and sphinganine. Total nucleic acid concentrations were measured as an index of cell number. Results are expressed as a percentage of the quantity at zero hours (mean ± standard error of the mean, n=3). *P<0.05 vs. the Ctr. Where an error bar is not seen, it lies within the dimensions of the symbol. Ctr, control; SO, sphingosine.

tumorigenic cells was detected using DNA fragmentation gel electrophoresis (Fig. 3A and B) and flow cytometry (Fig. 3C-G) assays. In Type I HBECs, control cultures exhibited intact, high molecular weight DNA that remained at the top of the agarose gel (Fig. 3A; lane 2), while DNA extracted from cells cultured with 10 μM C_2-ceramide for 1, 2 and 4 days was fragmented and demonstrated a characteristic DNA ladder pattern, indicative of apoptosis (Fig. 3A; lanes 3-5).

In tumorigenic cells, C_2-ceramide at 5 μM (Fig. 3B; lane 5) and 10 μM (Fig. 3B; lane 2) also caused the formation of a DNA ladder, an indicator of apoptosis. Flow cytometry analysis indicated that sphingosine (Fig. 3C-E) and C_2-ceramide (Fig. 3C, F and G) increased by ~5-7-fold the apoptotic cell populations (as demonstrated by A_0 peaks in the hypo-diploid pre-G_0/G_1 region).

Sphingosine, but not C_2-ceramide, induces differentiation of normal stem cells (Type I HBECs). Type I HBECs were cultured with sphingosine and C_2-ceramide at non-cytotoxic concentrations. Differentiation was measured by counting the numbers of Type II and Type I colonies that were morphologically distinguishable (Fig. 4).

As presented in Fig. 5 and Table I, control cells exhibited a low rate of differentiation of ~6.6% by day 5 and ~7.8% by day 9. Cholera toxin (1 ng/ml), a positive control known to induce differentiation (11,14), significantly increased the number of Type II containing colonies to 62% by day 5 and 93% by day 9 (P<0.001) without significant alteration in the total number of colonies. Sphingosine at non-cytotoxic concentrations (1 to 3 μM) significantly increased the frequency of Type II HBEC-containing colonies starting at day 5 (P<0.01; Fig. 5) compared with the vehicle control (0.1% BSA) culture. This trend became more obvious at day 9 (P<0.01; Fig. 5). The induction of differentiation of Type I to Type II HBECs by 2 and 3 μM sphingosine was accompanied by inhibition of colony forming efficiency, as presented by the decrease in the total number of colonies (Fig. 5 and Table I).

C_2-ceramide at 0.1 μM exhibited little effect on differentiation, while 0.3 and 0.5 μM inhibited differentiation compared with the vehicle control (0.1% ethanol) by day 8 (Fig. 6A). C_2-ceramide at 0.5 μM inhibited colony-forming efficiency, as indicated by a significant decrease in the number of total colonies (P<$5x10^{-5}$; Fig. 6A and Table II).

Since the vehicle control (0.1% ethanol) for C_2-ceramide treatments demonstrated a higher level of differentiation (Fig. 6A: Woman ID#1) compared with the vehicle control (0.1% BSA) for sphingosine treatments (Fig. 5), an additional independent experiment was conducted (Fig. 6B: Woman ID#2) to determine the effects of ethanol on the differentiation of Type I HBECs developed from reduction mammoplasty tissues of a different healthy (cancer-free) woman. Cholera toxin at 0.1 and 1 ng/ml was tested as a positive differentiation inducer of Type I HBECs (Fig. 6B). Although variations exist in % differentiation between different women and independent cell culture experiments, the fold-increases of differentiation induced by cholera toxin compared with those of controls for ceramide are comparable between woman ID#1 for day 8 (Fig 6A; Table II) and woman ID#2 for day 7 experiments (Fig. 6B; Table II). This suggests cholera toxin induced relatively similar levels of differentiation in two different normal Type I HBECs derived from two different women (Fig. 6). In this independent experiment (woman ID#2), percentages of differentiation of Type I HBECs were slightly increased in the 0.01 and 0.1% ethanol cultures compared with the corresponding control (nothing added) culture by day 7, however, these differences were not statistically significant (Fig. 6B and Table II).

Sphingosine decreases telomerase activity in tumorigenic cells. The effects of sphingosine on telomerase activity were examined (Fig. 7A) using a TRAP assay in tumorigenic cells. In this assay, telomerase-synthesized extension products were amplified by PCR and a ladder of products with 6 base increments, starting at 50 nucleotides, indicated positive

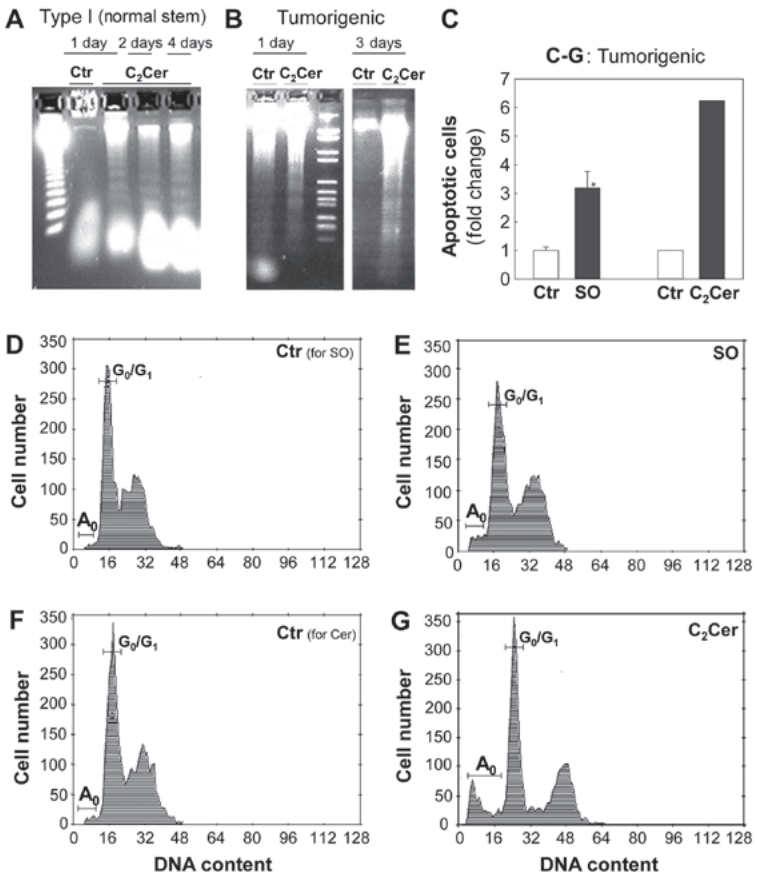

Figure 3. SO and C_2Cer induce apoptosis in tumorigenic cells. Apoptotic cells were detected by using DNA fragmentation gel electrophoresis (A and B) and flow cytometry (C-G) assays. (A and B) C_2Cer causes internucleosomal DNA fragmentation, indicative of apoptosis, in Type I HBECs (normal stem) and tumorigenic cells. DNA was extracted on indicated culture dates and analyzed via electrophoresis. (A) Type I HBECs were cultured with 10 μM C_2Cer for 1 day (lane 3), 2 days (lane 4), or 4 days (lane 5). Lane 1 presents DNA size markers and lane 2 is the Ctr at day 1. (B) Tumorigenic cells were cultured without C_2Cer (lanes 1 and 4), with 10 μM C_2Cer (lane 2) for 1 day, or with 5 μM C_2Cer (lane 5) for 3 days. Lane 3 presents DNA size markers. (C-G) SO and C_2Cer increase the number of apoptotic cells (A_0) at pre-G_0/G_1 regions in tumorigenic cells. Cells were stained with propidium iodide to determine DNA content and cell cycle via flow cytometry. (C) The effects of SO and C_2Cer on apoptosis of tumorigenic cells are presented by a fold-change in the number of apoptotic cells. Data are presented as the mean ± standard error of the mean (n=5) for SO from three independent culture experiments. *P<0.05 vs. the Ctr. Subconfluent tumorigenic cells were cultured with or without treatments for 1 day: (D) Ctr for SO; (E) 10 μM SO; (F) Ctr for C_2-Cer; (G) 10 μM C_2-Cer. Ctr, control; Cer, ceramide; SO, sphingosine; HBECs, human breast epithelial cells.

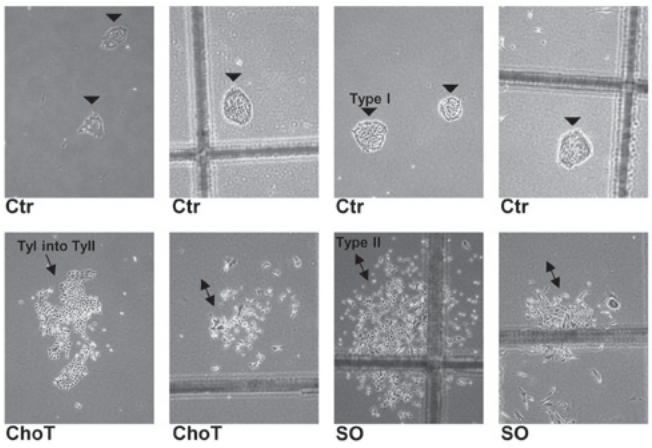

Figure 4. SO at non-cytotoxic concentrations induces differentiation of Type I HBECs to Type II HBECs (representative photographs at magnification x10 plus x4). Type I HBECs were seeded at 5,000 cells per 60 mm dish and grown for 2 days and then cultured with SO at 0.5 μM and ChoT at 1 ng/ml for 7 days. Fresh media with and without chemicals were renewed on days 3 and 5. The number of colonies of Type I (indicated with ▾), Type I differentiating into Type II (indicated by →) and Type II (indicated by ↔) HBECs were quantitated following 7 days treatment. SO, sphingosine; ChoT, cholera toxin; Ctr, control; HBECs, human breast epithelial cells.

telomerase activity. An internal control was included to quantify telomerase activity and identify false negative results.

Intensities of telomerase-synthesized/PCR-amplified DNA extension ladders were quantified and presented in Fig. 7B and C. The comparable internal standard indicates similar amplification efficiency in both control and sphingosine-treated groups (Fig. 7A). Sphingosine at 5 μM significantly reduced telomerase activity by ~1.5-fold within 2 days ($P<0.001$; Fig. 7) in comparison to the control.

Discussion

A unique aspect of the current study is that it evaluated the plausibility of using sphingolipid metabolites as chemotherapeutic drugs by comparing their effects on the proliferation and death of tumorigenic breast cells to normal breast differentiated (Type II) cells. Furthermore, since Type I HBECs have stem cell characteristics and are more susceptible to neoplastic transformation (16), the present study also evaluated the cancer-protective potential of sphingosine and C_2-ceramide by examining their effects on proliferation, death, and differentiation of Type I HBECs.

Table II. Effects of C_2Cer and ethanol on differentiation of Type I HBECs.

Culture (days)	Treatments	Total Total colonies	Type II-containing colonies	% of Type II-containing colonies (95% CI)	Fold-change (ChoT/ Ctr for Cer)
Type I HBECs from woman ID#1					
8	Ethanol 0.1% (Ctr for Cer)	517	125	24.2% (-3.5%)	
	ChoT 1 ng/ml	462	250	54.1% (-4.6%)[a]	↑ 2.24
	C_2Cer 0.1 μM	503	105	20.9% (-3.3%)	
	C_2Cer 0.3 μM	466	52	11.2% (-2.6%)[a]	
	C_2Cer 0.5 μM	138	4	2.9% (-1.8%)[a]	
Type I HBECs from woman ID#2					
7	Control (nothing added)	98	6	6.1% (-3.3%)	
	Ethanol 0.01%	151	20	13.3% (-4.5%)	
	Ethanol 0.1% (Ctr for Cer)	107	11	10.3% (-4.4%)	
	ChoT 0.1 ng/ml	99	23	23.2% (-7.2%)	↑ 2.26
	ChoT 1 ng/ml	95	17	17.9% (-6.4%)	↑ 1.74

Type I HBECs ($5x10^3$) were cultured in 60 mm dishes with grids in triplicate and treated with C_2Cer at non-cytotoxic concentrations (0.1 to 0.5 μM) and ethanol at 0.01 and 0.1%. ChoT was used as a positive differentiation inducer. Ethanol at 0.1% was used as vehicle control for C_2Cer treated cells. Independent experiments were conducted by culturing Type I HBECs developed from breast tissues of two different healthy (cancer-free) women ID#1 and #2. Type I and Type II colonies were quantified at indicated culture dates. The % differentiation is calculated by (number of Type II HBEC-containing colonies/total number of colonies) x100. [a]$P<5x10^{-5}$; (by Chi-square test with Bonferonni corrections) vs. Ctr. The lower bounds of Wilson score 95% CI are indicated in parentheses. CI, confidence intervals; Cer, ceramide; HBECs, human breast epithelial cells.

A major result of the present study is that sphingosine preferentially inhibits proliferation and causes death of normal breast stem cells (Type I HBECs) and tumorigenic cells, while having little or no effect on the proliferation of normal differentiated breast cells (Type II HBECs). Type II HBECs were used as normal cell counterparts of breast cancer cells. The fact that sphingosine selectively inhibits tumorigenic cells but has little effect on Type II HBECs implies that sphingosine may be an ideal therapeutic agent with low potential for side effects. Furthermore, since apoptosis induced by sphingosine is a well-regulated process, sphingosine is likely to have the advantage of targeting cancer cells without eliciting an inflammatory response in the surrounding normal tissue.

Tumorigenic breast cells, MCF7 breast cancer cells, and Type I HBECs (normal stem) demonstrated similar sensitivity to anti-proliferative and pro-apoptotic effects of sphingosine. This may be because the phenotypes of breast tumorigenic cells (M13SV1R2N1) and breast cancer cells (e.g. MCF7 and MDA-MB-231) have been demonstrated to be more similar to those of Type I HBECs rather than Type II HBECs (10-12). These similar sensitivities of Type I HBECs, tumorigenic cells, and MCF7 cancer cells to the effects of sphingosine could be evidence for the cancer stem cell hypothesis (19).

Ceramides with an N-acyl chain shorter than 8 to 10 carbon atoms are generally called 'short-chain ceramides'. Since most naturally occurring ceramides cannot be dispersed in water, due to their hydrophobicity, short-chain ceramides and, in particular, C_2-ceramide, have been extensively used as agonists when ceramide effects need to be produced, both in intact cells and in cell-free systems (20). In the present study, effects of C_2-ceramide were compared with those of sphingosine. Unlike sphingosine, C_2-ceramide does not demonstrate the selective anti-proliferative effects. C_2-ceramide inhibits proliferation of normal differentiated cells, tumorigenic cells, and MCF7 cancer cells with similar potency. Although C_2-ceramide seems to be more potent compared with sphingosine in that 5 μM C_2-ceramide causes similar cytotoxic effects as 8 to 10 μM sphingosine, C_2-ceramide does not appear to be an ideal chemotherapeutic agent due to its anti-proliferative and cytotoxic effects on normal differentiated cells (Type II HBECs). Mechanisms are unclear for nonselective anti-proliferative effects of a short-chain cell-permeable ceramide, including C_2-ceramide, in normal differentiated cells. Goñi *et al* (21) demonstrated that biophysical properties of short-chain ceramides are affected by their different N-acyl chain lengths. N-acyl chain length also affects flip-flop lipid motion (22). Taken together, the presence of N-acyl chain in C_2-ceramide may affect cellular uptake, membrane permeabilities, or binding specific sites in the target proteins, which may account for different effects on proliferation of Type II HBECs in comparison to sphingosine.

The results of the present study indicate that unnatural D-*threo*-, L-*threo*-, and L-*erythro*-stereoisomers of sphingosine are more potent compared with natural D-*erythro*-sphingosine in inhibiting proliferation and causing death in breast tumorigenic cells. In contrast, L-*erythro* and DL-threo-sphingosine isomers did not induce apoptosis in RD embryonal rhabdomyosarcoma cell line, while D-*erythro*-sphingosine induced apoptosis (23). This suggests that the stereo-specific efficacy of sphingosine isomers on proliferation or apoptosis may be influenced by different cellular and genetic backgrounds of tumor origins, which could affect the uptake and metabolism of sphingosine and unnatural sphingosine isomers.

Another major result of the present study is that non-cytotoxic (sub-lethal) concentrations of sphingosine induce differentiation of Type I (normal stem) to Type II HBECs, thereby reducing the number of stem cells, which are the target cells of carcinogenesis. C_2-ceramide, however, did not induce differentiation of Type I to Type II HBECs at non-cytotoxic concentrations. This suggests that sphingosine can be a potential cancer-protective agent, whereas C_2-ceramide does not appear to be a cancer-protective agent due to its inability of inducing the differentiation of Type I to Type II HBECs and its anti-proliferative effects on Type II HBECs.

The roles of ethanol in proliferation and differentiation are not clearly understood. The effects of ethanol may vary depending on concentration, origins of cell types, culture conditions, or stages of cellular immortalization and neoplastic transformation. Ethanol enhanced neural differentiation of PC12 rat pheochromocytoma cells with the involvement of protein kinase C (24). Ethanol inhibited skeletal muscle cell

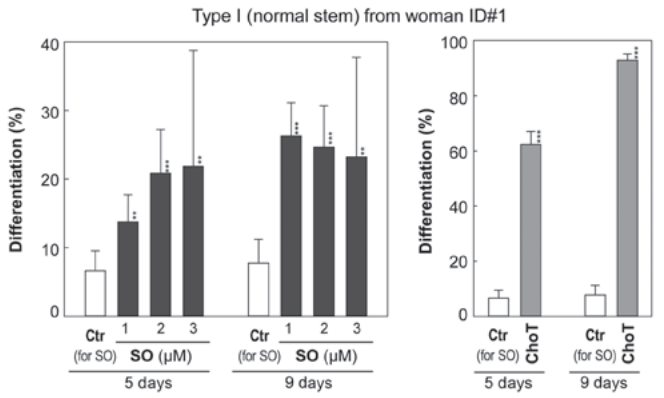

Figure 5. SO at non-cytotoxic concentrations induces differentiation of Type I HBECs to Type II HBECs. Type I HBECs derived from woman ID#1 were seeded at 5,000 cells/60 mm dishes with grids in triplicate and grown for 2 days in the 'MSU-1+S medium' with 5% fetal bovine serum and then cultured with SO in the 'MSU-1+S medium' for 5 or 9 days. Vehicle control (0.1% bovine serum albumin) was used for SO (1 to 3 μM) treated cells. ChoT (1 ng/ml) was used as a positive differentiation inducer. The % differentiation is calculated by [number of Type II HBECs containing colonies/total number of colonies] x100. Error bars represent the upper bounds of Wilson score 95% confidence intervals. Significant differences compared with the corresponding controls are indicated. **P<0.01 and ***P<0.001 vs. the Ctr (by Chi-square test with Bonferroni corrections). ChoT, cholera toxin; SO, sphingosine; HBECs, human breast epithelial cells; Ctr, control.

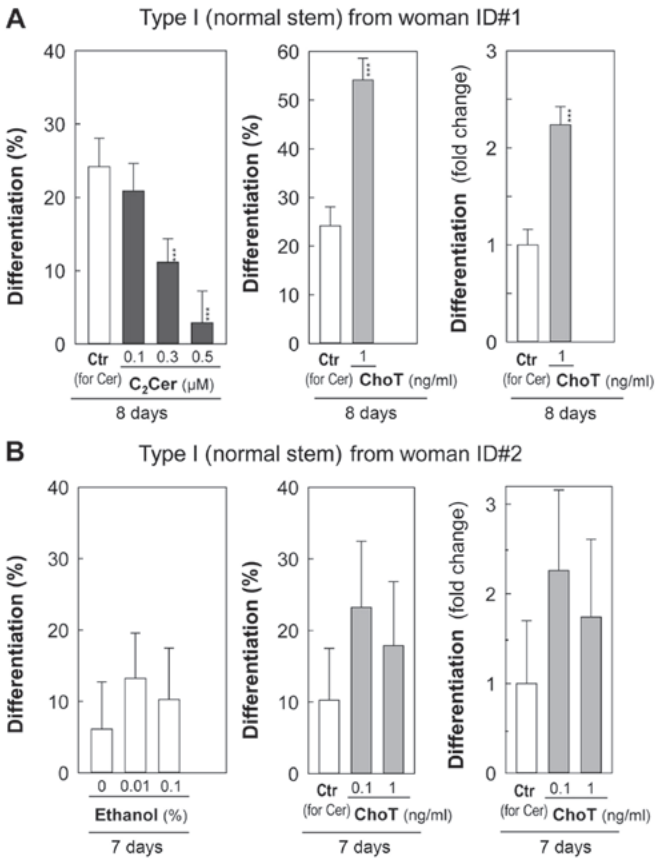

Figure 6. C_2Cer at non-cytotoxic concentrations does not induce differentiation of Type I HBECs. Type I HBECs were seeded at 5,000 cells/60 mm dishes with grids in triplicate and grown for 2 days in the 'MSU-1+S medium' with 5% fetal bovine serum and then cultured with treatments in the 'MSU-1+S medium' for 7 or 8 days. ChoT (0.1 and 1 ng/ml) was used as a positive differentiation inducer. The % differentiation is calculated by (number of Type II HBEC containing colonies/total number of colonies) x100. Error bars represent the upper bounds of Wilson score 95% confidence intervals. (A and B) ***P<0.001 (by Chi-Square test with Bonferroni corrections) for a concentration of a treatment (C_2-Cer, ChoT or Ethanol) vs. the Ctr. Ethanol at 0.1% was used as vehicle control for C_2-Ceramide treated cells. Independent experiments were conducted by culturing Type I HBECs developed from breast tissues of two different healthy (cancer-free) women ID#1 (A) and #2 (B). ChoT, cholera toxin; HBECs, human breast epithelial cells; Cer, ceramide.

proliferation and delays its differentiation in cell culture (25). Ethanol at 0.3% stimulated proliferation of MCF7 human breast cancer cells, while ethanol at higher than 0.3% inhibited the proliferation (26). This stimulation of proliferation by ethanol was mediated by elevated activity of extracellular signal-regulated kinase-1/2 in MCF7 cells (26). Ethanol at 10^{-3} M increased proliferation and reduced differentiation of head and neck squamous carcinoma cells (27). In the present study, although it is not statistically significant, ethanol at 0.01 and 0.1%, which are lower concentrations than most previous ethanol studies examined, demonstrated a tendency of inducing differentiation of normal Type I HBECs. A future study which focuses on ethanol can examine the effects of ethanol at a broader range of concentrations on normal stem cells derived from breast tissues of additional subjects to more firmly establish the roles of ethanol on differentiation.

The mechanism by which sphingosine induces differentiation of Type I (normal stem) HBECs is not clear. Most differentiation studies have examined the effects of sphingosine-1-phosphate (S1P) or various chain length analogs of ceramide. Less is known about effects of sphingosine on differentiation of cancer cells or normal cells, particularly on normal stem cells. Hui *et al* (28) demonstrated that sphinganine facilitated the retinoic acid induced differentiation of HL60 promyelocytic leukemia cells. In a study by Ohta *et al* (29), differentiation induced by 4 β-phorbol 12-myristate 13-acetate increased cellular sphingosine levels in HL60 cells. Sphingosine levels in the cells increased concurrently with the increasing proportion of apoptotic cells during cell differentiation (29). Sphingosine treatment induced apoptosis and downregulated *c-myc* mRNA expression in HL60 cells (29). This suggests that sphingosine may induce differentiation by regulating *c-myc* expression. Cholera toxin, a well-known inducer of cAMP, induced the differentiation of Type I to Type II HBECs in the authors' previous (11,14) and current studies. S1P, which displays the opposite effects to sphingosine, inhibited differentiation of placental trophoblasts (30) and adipogenic differentiation of C3H10T1/2 murine mesenchymal stem cells (31) by reducing intracellular cAMP accumulation. This suggests that sphingosine induced-differentiation of Type I HBECs may involve a mechanism that increases intracellular cAMP.

Telomerase, the ribonucleoprotein reverse transcriptase that maintains the ends of human chromosomes (telomeres), is activated in the majority of breast cancer types but not in normal adjacent tissues (32) and its activity is associated with poor prognosis and more aggressive phenotypes (33). Therefore, telomerase is an attractive therapeutic target and can be a useful biomarker for breast cancer diagnosis and prognosis. In the present study, it was demonstrated that sphingosine at growth inhibitory concentrations decreased telomerase activity in breast tumorigenic cells. Several mechanisms may explain how sphingosine reduces telomerase activity. First, *c-myc* has

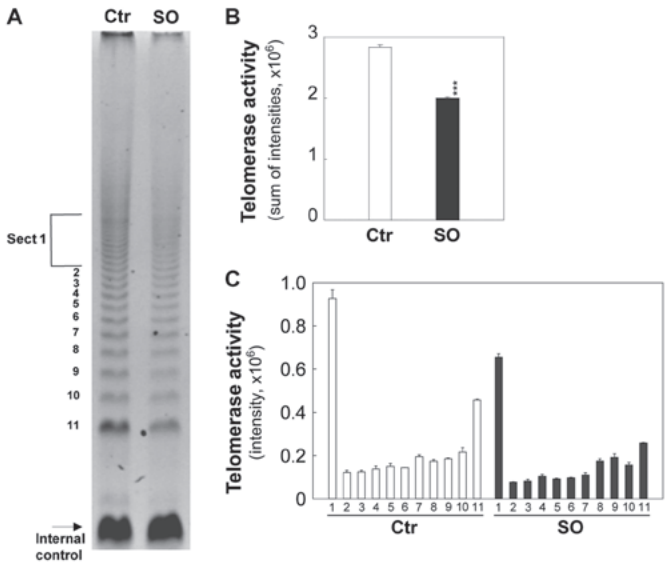

Figure 7. SO inhibits telomerase activity in tumorigenic cells. Subconfluent cells were incubated without (lane 1) or with 5 μM SO (lane 2) for 2 days. Collected cells were analyzed by the telomerase repeat amplification protocol assay. (A) The PCR-amplified telomerase products were electrophoresed in 12% polyacrylamide gel. Telomerase activity is visualized by the characteristic 6 base pair ladders and normalized by internal control. The first lane (Ctr) and the second lane (5 μM SO) are split into 11 sections, respectively. Bar 1 representing the intensity of section 1 of the PCR products on the polyacrylamide gel, bar 2 representing 2, and so on through bar 11. (B and C) The electrophoresed PCR-amplified telomerase products' intensities, normalized by the internal control, are quantified via densitometry. The sum of these telomerase products' intensities is demonstrated in (B). ***P<0.001 vs. Ctr (by t-test). Each intensity of these PCR product sections is demonstrated in (C). Where an error bar is not seen, it lies within the dimensions of the symbol. Ctr, control; SO, Sphingosine; PCR, polymerase chain reaction.

been demonstrated to activate the transcription of telomerase reverse transcriptase in Epstein Barr virus-immortalized B cells engineered to express c-MYC-activated or c-MYC-repressed genes (34). Second, sphingosine induces apoptosis and downregulates *c-myc* expression in HL60 cells (29). Therefore, sphingosine may reduce telomerase activity by downregulating *c-myc* expression (29). Among protein kinase C inhibitors tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporine demonstrated a moderate inhibition, and sphingosine produced a small inhibition of telomerase activity (35). Sphingosine inhibits protein kinase C activity *in vitro* (36) and in glioma C6 cells (37). Therefore, sphingosine may suppress telomerase activity by inhibiting protein kinase C in breast tumorigenic cells. Third, a close correlation exists between differentiation and the suppressed activity of telomerase. For example, the inhibition of telomerase activity was a common response to the induction of differentiation in HL60, K562, F9, and CCE24 cells by dimethyl sulfoxide, retinoic acid, vitamin D3, or 12-*0*-tetradecanoyl-1-phorbol-13-acetate (38) and during the monocytic or granulocytic differentiation of HL60 cells by vitamin D3, all-trans retinoic acid, and Am80 (39). This suggests that sphingosine may induce the differentiation of breast tumorigenic cells by inhibiting telomerase activity.

In conclusion, the chemotherapeutic and cancer-protective properties of sphingosine and C_2-ceramide for breast cancer were evaluated by examining their effects on proliferation, apoptosis, and differentiation of normal stem, normal differentiated, and tumorigenic breast cells. The results indicate that sphingosine: i) preferentially inhibits proliferation and causes death of Type I (normal stem) HBECs and tumorigenic breast cells but has little effect on Type II (normal differentiated) HBECs; ii) induces apoptosis in breast tumorigenic cells; iii) induces differentiation of Type I to Type II HBECs at non-cytotoxic concentrations; and iv) inhibits telomerase activity in breast tumorigenic/cancer cells. These results suggest that sphingosine has the potential to be used as a chemotherapeutic and cancer-protective agent for human breast cancer. C_2-ceramide demonstrates comparable growth-inhibitory effects on proliferation of Type I HBECs and tumorigenic breast cells by inducing apoptosis. However, C_2-ceramide is cytotoxic to Type II HBECs and fails to induce differentiation of Type I to Type II HBECs. Therefore, C_2-ceramide does not appear to be an ideal chemotherapeutic or cancer-protective agent for human breast cancer.

Acknowledgements

The authors thank Ms. Susan Kim for graphical assistance, Mr. Howard Nebeck for proofreading the manuscript, and Dr Normal Hord for comments on this manuscript.

Funding

The present study was supported by U.S. Army Medical Research and Material Command under DAMD17-96-1-6099 to CCC., Michigan State University Agricultural Experiment Station to JJS., National Institute of Environmental Health Sciences (NIEHS) sponsored University of Washington Center for Ecogenetics and Environmental Health and ITHS grant no. NIH/NIEHS P30ES007033 to EHA and University of Washington Office of Research Royalty Research Fund grant to EHA.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Conceived and designed the experiments: HY, EHA, CCC, JJS. Performed the experiments: HY, EHA, CYH, WS, CCC. Analyzed the data: EHA, HY. Contributed reagents/materials/analysis tools: EHA, HY, CCC, JJS. Wrote the paper: EHA, HY, CCC, JJS.

Ethics approval and consent to participate

Patients' written consent was obtained in accordance with institutional guidelines for obtaining breast tissues during reduction mammoplasty. Procedures were approved by Sparrow Hospital (Lansing, MI, USA) Institutional Review Board and Michigan State University Human Research Projection Program.

Patient consent for publication

Patients' written consent was obtained in accordance with institutional guidelines for obtaining breast tissues during reduction mammoplasty.

Competing interests

The authors declare that they have no competing interests.

References

1. American Cancer Society: Cancer Facts and Figures 2017. American Cancer Society Inc., Atlanta, GA, 2017.
2. Eheman CR, Shaw KM, Ryerson AB, Miller JW, Ajani UA and White MC: The changing incidence of in situ and invasive ductal and lobular breast carcinomas: United States, 1999-2004. Cancer Epidemiol Biomarkers Prev 18: 1763-1769, 2009.
3. Russo J and Russo IH: Biological and molecular bases of mammary carcinogenesis. Lab Invest 57: 112-137, 1987.
4. Oakes SR, Gallego-Ortega D and Ormandy CJ: The mammary cellular hierarchy and breast cancer. Cell Mol Life Sci 71: 4301-4324, 2014.
5. Ahn EH and Schroeder JJ: Bioactive sphingolipids are significant constituents of soy and dairy products. J Food Sci 67: 522-524, 2002.
6. Hannun YA and Obeid LM: Principles of bioactive lipid signalling: Lessons from sphingolipids. Nat Rev Mol Cell Biol 9: 139-150, 2008.
7. Ogretmen B: Sphingolipid metabolism in cancer signalling and therapy. Nat Rev Cancer 18: 33-50, 2018.
8. Huang YL, Huang WP and Lee H: Roles of sphingosine 1-phosphate on tumorigenesis. World J Biol Chem 2: 25-34, 2011.
9. Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK and Trosko JE: Oct4 expression in adult human stem cells: Evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 26: 495-502, 2005.
10. Kang KS, Morita I, Cruz A, Jeon YJ, Trosko JE and Chang CC: Expression of estrogen receptors in a normal human breast epithelial cell type with luminal and stem cell characteristics and its neoplastically transformed cell lines. Carcinogenesis 18: 251-257, 1997.
11. Kao CY, Nomata K, Oakley CS, Welsch CW and Chang CC: Two types of normal human breast epithelial cells derived from reduction mammoplasty: Phenotypic characterization and response to SV40 transfection. Carcinogenesis 16: 531-538, 1995.
12. Kao CY, Oakley CS, Welsch CW and Chang CC: Growth requirements and neoplastic transformation of two types of normal human breast epithelial cells derived from reduction mammoplasty. In Vitro Cell Dev Biol Anim 33: 282-288, 1997.
13. Chang CC, Sun W, Cruz A, Saitoh M, Tai MH and Trosko JE: A human breast epithelial cell type with stem cell characteristics as target cells for carcinogenesis. Radiat Res 155: 201-207, 2001.
14. Ahn EH, Chang CC and Schroeder JJ: Evaluation of sphinganine and sphingosine as human breast cancer chemotherapeutic and chemopreventive agents. Exp Biol Med (Maywood) 231: 1664-1672, 2006.
15. Kang KS, Sun W, Nomata K, Morita I, Cruz A, Liu CJ, Trosko JE and Chang CC: Involvement of tyrosine phosphorylation of p185(c-erbB2/neu) in tumorigenicity induced by X-rays and the neu oncogene in human breast epithelial cells. Mol Carcinog 21: 225-233, 1998.
16. Sun W, Kang KS, Morita I, Trosko JE and Chang CC: High susceptibility of a human breast epithelial cell type with stem cell characteristics to telomerase activation and immortalization. Cancer Res 59: 6118-6123, 1999.
17. Ahn EH and Schroeder JJ: Sphingoid bases and ceramide induce apoptosis in HT-29 and HCT-116 human colon cancer cells. Exp Biol Med (Maywood) 227: 345-353, 2002b.
18. Chang CC: Recent translational research: Stem cells as the roots of breast cancer. Breast Cancer Res 8: 103, 2006.
19. Kakarala M and Wicha MS: Implications of the cancer stem-cell hypothesis for breast cancer prevention and therapy. J Clin Oncol 26: 2813-2820, 2008.
20. Goñi FM and Alonso A: Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. Biochim Biophys Acta 1758: 1902-1921, 2006.
21. Goñi FM, Contreras FX, Montes LR, Sot J and Alonso A: Biophysics (and sociology) of ceramides. Biochem Soc Symp 72: 177-188, 2005.
22. Contreras FX, Basañez G, Alonso A, Herrmann A and Goñi FM: Asymmetric addition of ceramides but not dihydroceramides promotes transbilayer (flip-flop) lipid motion in membranes. Biophys J 88: 348-359, 2005.
23. Phillips DC, Martin S, Doyle BT and Houghton JA: Sphingosine-induced apoptosis in rhabdomyosarcoma cell lines is dependent on pre-mitochondrial Bax activation and post-mitochondrial caspases. Cancer Res 67: 756-764, 2007.
24. Messing RO: Ethanol as an enhancer of neural differentiation. Alcohol Alcohol Suppl 2: 289-293, 1993.
25. Garriga J, Adanero E, Fernández-Solá J, Urbano-Márquez A and Cussó R: Ethanol inhibits skeletal muscle cell proliferation and delays its differentiation in cell culture. Alcohol Alcohol 35: 236-241, 2000.
26. Izevbigie EB, Ekunwe SI, Jordan J and Howard CB: Ethanol modulates the growth of human breast cancer cells in vitro. Exp Biol Med (Maywood) 227: 260-265, 2002.
27. Kornfehl J, Temmel A, Formanek M and Knerer B: Effects of ethanol treatment of proliferation and differentiation in a head and neck squamous cell carcinoma cell line. Alcohol Clin Exp Res 23: 1102-1107, 1999.
28. Hui EK, Yang YH and Yung BY: Schedule-dependent sphinganine potentiation of retinoic acid-induced differentiation, cell growth inhibition, and nucleophosmin translocation in a human leukemia cell line (HL-60). Exp Hematol 20: 454-461, 1992.
29. Ohta H, Sweeney EA, Masamune A, Yatomi Y, Hakomori S and Igarashi Y: Induction of apoptosis by sphingosine in human leukemic HL-60 cells: A possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. Cancer Res 55: 691-697, 1995.
30. Johnstone ED, Chan G, Sibley CP, Davidge ST, Lowen B and Guilbert LJ: Sphingosine-1-phosphate inhibition of placental trophoblast differentiation through a G(i)-coupled receptor response. J Lipid Res 46: 1833-1839, 2005.
31. Hashimoto Y, Matsuzaki E, Higashi K, Takahashi-Yanaga F, Takano A, Hirata M and Nishimura F: Sphingosine-1-phosphate inhibits differentiation of C3H10T1/2 cells into adipocyte. Mol Cell Biochem 401: 39-47, 2015.
32. Herbert BS, Wright WE and Shay JW: Telomerase and breast cancer. Breast Cancer Res 3: 146-149, 2001.
33. Kulić A, Plavetić ND, Gamulin S, Jakić-Razumović J, Vrbanec D and Sirotković-Skerlev M: Telomerase activity in breast cancer patients: Association with poor prognosis and more aggressive phenotype. Med Oncol 33: 23, 2016.
34. Wu KJ, Grandori C, Amacker M, Simon-Vermot N, Polack A, Lingner J and Dalla-Favera R: Direct activation of TERT transcription by c-MYC. Nat Genet 21: 220-224, 1999.
35. Ku WC, Cheng AJ and Wang TC: Inhibition of telomerase activity by PKC inhibitors in human nasopharyngeal cancer cells in culture. Biochem Biophys Res Commun 241: 730-736, 1997.
36. Hannun YA, Loomis CR, Merrill AH Jr and Bell RM: Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. J Biol Chem 261: 12604-12609, 1986.
37. Czajkowski R and Barańska J: Sphingosine and phorbol ester modulate protein kinase C activity and modify ATP-evoked calcium mobilization in glioma C6 cells. Biochem Biophys Res Commun 260: 614-618, 1999.
38. Sharma HW, Sokoloski JA, Perez JR, Maltese JY, Sartorelli AC, Stein CA, Nichols G, Khaled Z, Telang NT and Narayanan R: Differentiation of immortal cells inhibits telomerase activity. Proc Natl Acad Sci USA 92: 12343-12346, 1995.
39. Yamada O, Ozaki K, Nakatake M, Akiyama M, Kawauchi K and Matsuoka R: Multistep regulation of telomerase during differentiation of HL60 cells. J Leukoc Biol 83: 1240-1248, 2008.