Evaluation of chemotherapeutic and cancer-protective properties of sphingosine and C₂-ceramide in a human breast stem cell derived carcinogenesis model

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Abstract. The overall goal of the present study was to evaluate the chemotherapeutic and cancer-protective properties of D-erythro-sphingosine (sphingosine) and C₂-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, C₂-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 C₂-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 C₂-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 C₂-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.

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.

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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 C2-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 C2-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 C2- 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.), 10^5 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), 0.01% 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 cymometer (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 (Ap peaks) with reduced DNA content were determined as the percentage of cells in the hypo-diploid sub G0/G1 area to the left of G0/G1 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 sphingolipid groups at a single concentration for: Apoptotic cells at pre-G0/G1 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.

C2-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), C2-ceramide at 2 and 5 µM significantly
inhibited cell proliferation (P<0.05) and caused death within 1 day. C₂-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 3. Subconfluent cells (6x10⁴) 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 C₂-Cer (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.

The unnatural stereoisomers of sphingosine more potently inhibit proliferation and cause death of 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 C₂-ceramide induce apoptosis in normal stem cells (Type I) and tumorigenic cells. To examine whether sphingosine and C₂-ceramide caused cell death by inducing apoptosis, the presence of apoptotic cells in Type I HBECs and

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, C₂-Cer 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 (6x10⁴) 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 C₂-Cer (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.
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) |
|---------------|------------|----------------|----------------------------|----------------------------------------|
| 5             | Ctr for SO (0.1% BSA) | 392            | 26                         | 6.6% (-2.1%)                           |
|               | Cholera toxin 1 ng/ml | 371            | 231                        | 62.3% (-5.0%)                          |
|               | SO 1 µM      | 356            | 49                         | 13.8% (-3.2%)                          |
|               | SO 2 µM      | 187            | 39                         | 20.9% (-5.2%)                          |
|               | SO 3 µM      | 32             | 7                          | 21.9% (-10.9%)                         |
| 9             | Ctr for SO (0.1% BSA) | 322            | 25                         | 7.8% (-2.5%)                           |
|               | Cholera toxin 1 ng/ml | 335            | 311                        | 92.8% (-3.3%)                          |
|               | SO 1 µM      | 350            | 92                         | 26.3% (-4.3%)                          |
|               | SO 2 µM      | 223            | 55                         | 24.7% (-5.2%)                          |
|               | SO 3 µM      | 43             | 10                         | 23.3% (-10.1%)                         |

Type I HBECs (5x10⁴) were cultured in 60 mm dishes with grids in triplicate and treated with SO at non-cytotoxic concentrations (1 to 3 µM). Cholera toxin 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. *P<0.01; †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; 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 (6x10⁴) were cultured in 6-well plates in triplicate and treated with 5 µM SO stereoisomers and sphingosine. 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 C₂-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, C₂-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 C₂-ceramide (Fig. 3C, F and G) increased by ~5-7-fold the apoptotic cell populations (as demonstrated by A₀ peaks in the hypo-diploid pre-G₀/G₁ region).

Sphingosine, but not C₂-ceramide, induces differentiation of normal stem cells (Type I HBECs). Type I HBECs were cultured with sphingosine and C₂-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).

C₂-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). C₂-ceramide at 0.5 µM inhibited colony-forming efficiency, as indicated by a significant decrease in the number of total colonies (P<5x10⁻³; Fig. 6A and Table II).

Since the vehicle control (0.1% ethanol) for C₂-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 mammaryplasty 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
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 C₂-ceramide by examining their effects on proliferation, death, and differentiation of Type I HBECs.
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 potential to induce apoptosis in the target 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, C2-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 C2-ceramide were compared with those of sphingosine. Unlike sphingosine, C2-ceramide does not demonstrate the selective anti-proliferative effects. C2-ceramide inhibits proliferation of normal differentiated cells, tumorigenic cells, and MCF7 cancer cells with similar potency. Although C2-ceramide seems to be more potent compared with sphingosine in that 5 µM C2-ceramide causes similar cytotoxic effects as 8 to 10 µM sphingosine, C2-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 C2-ceramide, in normal differentiated cells. Goshi 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 C2-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. C2-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 C2-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

### Table II. Effects of C2-Cer and ethanol on differentiation of Type I HBECs

| Culture (days) | Treatments | Total 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%) | 1.74 |
| | ChoT 1 ng/ml | 462 | 250 | 54.1% (-4.6%) | + 2.26 |
| | C2-Cer 0.1 µM | 503 | 105 | 20.9% (-3.3%) | + 1.74 |
| | C2-Cer 0.3 µM | 466 | 52 | 11.2% (-2.6%) | + 2.24 |
| | C2-Cer 0.5 µM | 138 | 4 | 2.9% (-1.8%) | + 2.26 |
| 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%) | + 2.26 |

Type I HBECs (5x10^2) were cultured in 60 mm dishes with grids in triplicate and treated with C2-Cer 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 C2-Cer 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. *P<5x10^-5; (by Chi-square test with Bonferroni 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.
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 reinitic 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 expression of the opposite effects to sphingosine, inhibited differentiation of placental trophoblasts (30) and adipogenic differentiation of C3H10T1/2 murine mesenchymal stem cells (31) by reducing expression of c-myc. AHN et al: ROLES OF SPHINGOSINE AND C2-CERAMIDE IN BREAST CANCER

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] x 100. 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, choler toxin; SO, sphingosine; HBECs, human breast epithelial cells; Ctr, control.

Figure 6. C2-Ceramide 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) x 100. 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 (C2-Cer, ChoT or Ethanol) vs. the Ctr. Ethanol at 0.1% was used as vehicle control for C2-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, choler toxin; HBECs, human breast epithelial cells; Cer, ceramide.
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 down-regulates 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, bisindolylma-leimide I and H-7 demonstrated a big inhibition, staurosporine tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporine tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporine tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporine tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporine tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporine tested in human nasopharyngeal cancer cells, bisindolylmaleimide I and H-7 demonstrated a big inhibition, staurosporin...
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.

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