Evaluation of Sphinganine and Sphingosine as Human Breast Cancer Chemotherapeutic and Chemopreventive Agents

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No comparative study of the effects of sphingolipid metabolites on proliferation and differentiation in normal human breast epithelial cells versus stem cells and tumorigenic cells has been reported. The purpose of this study was to evaluate the chemotherapeutic and chemopreventive potential of sphingoid bases (sphingosine and sphinganine) using a novel cell culture system of normal human breast epithelial cells (HBEC) developed from breast tissues of healthy women obtained during reduction mammoplasty (Type I HBEC with stem cell characteristics and Type II HBEC with basal epithelial cell phenotypes) and transformed tumorigenic Type I HBEC. The results show that sphinganine inhibited the growth and induced apoptosis of transformed tumorigenic Type I HBEC more potently than sphingosine (IC_{50} for sphinganine 4 μM; sphingosine 6.4 μM). Both sphinganine and sphingosine at high concentrations (8–10 μM) arrested the cell cycle at G_2/M. Sphinganine inhibited the growth and caused death of Type I HBEC more strongly than sphingosine. In comparison, Type II HBEC (normal differentiated cells) were less sensitive to the growth-inhibitory effects of sphingoid bases than Type I HBEC (stem cells) or transformed tumorigenic Type I HBEC, suggesting that sphingoid bases may serve as chemotherapeutic agents. At concentrations (0.05, 0.1, and 0.5 μM) that are below the growth-inhibitory range, sphingoid bases induced differentiation of Type I HBEC to Type II HBEC, as detected morphologically and *via* expression of a tumor suppressor protein, maspin, which is a marker of Type II HBEC. Thus, sphingoid bases may function as chemotherapeutic as well as chemopreventive agents by preferentially inhibiting cancer cells and eliminating stem cells from which most breast cancer cells arise. Exp Biol Med 231:1664–1672, 2006

Key words: sphinganine; sphingosine; breast cancer; stem cells; apoptosis; differentiation

Introduction

Breast cancer is the most common cancer among women in the United States and worldwide (1, 2). The search for and development of chemotherapeutic and/or chemopreventive agents that do not exert side effects and drug resistance has been an active area of research.

Sphingolipids are found in all eukaryotic cell membranes, some prokaryotes, and also in a variety of foods, including dairy and soy products (3, for review, see Ref. 4). There is evidence that at least some portion of dietary sphingolipids are digested in the gastrointestinal tract and absorbed as sphingoid bases (sphingosine and sphinganine) and, perhaps, ceramides (5–7). Small amounts of free sphingoid bases are present in blood (8, 9). Abnet *et al.* (10) reported that median serum concentrations for the study population (265 residents of Linxian, People's Republic of China) were 52 nmol/L for sphinganine and 64 nmol/L for sphingosine.

Ceramide (acylated form of sphingosine) and sphingoid bases function as second messengers in signal transduction pathways, inhibiting cell proliferation and inducing apoptosis (programmed cell death) in human cancer cells (11–14), including colon (11) and breast cancer cells (13, 14). Moreover, complex dietary sphingolipids, including sphingomyelin, dihydrosphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD_3, reduced aberrant colonic foci in CF1 mice treated with 1,2-dimethylhydrazine (DMH) (15–20) and the number of tumors in all regions

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of the intestine in Multiple Intestinal Neoplasia (Min) mice with a truncated Adenomatous Polyposis Coli (APC) gene product (20, 21).

Most studies examining sphingolipids have focused on the proapoptotic action of ceramide and fewer studies have examined the action of sphingosine and sphinganine. It is uncertain whether the sphingosine (with the 4,5-*trans* double bond) or sphinganine (without the 4,5-*trans* double bond) backbone of ceramides mediates antiproliferative and proapoptotic activities of ceramide in cancer cells (22, 23; for review, see Refs. 24, 25). Furthermore, no study has compared the effects of sphingoid bases on proliferation and differentiation in human normal epithelial cells to that in human epithelial stem cells and tumorigenic cells derived from stem cells.

Previously, we have reported the characterization of two types of morphologically distinguishable and phenotypically different normal human breast epithelial cells (HBEC) from reduction mammoplasty tissues of healthy females (Type I and Type II HBEC) (26; for review, see Ref. 27). Type I HBEC display stem cell characteristics (i.e., the deficiency in gap-junctional intercellular communication [26], the ability of Type I HBEC to differentiate into Type II HBEC [26] and to form budding/ductal organoids on Matrigel in conjunction with Type II HBEC [27]). Type I HBEC express estrogen receptor α (28) and are more susceptible to telomerase activation, immortalization, and neoplastic transformation (26, 29–31). Furthermore, Type I HBEC were sequentially transformed to immortal/nontumorigenic cells (M13SV1), weakly tumorigenic cells (M13SV1R2), and highly tumorigenic cells (M13SV1R2N1) by treatments with the SV40 large T-antigen, X-rays, and neu oncogene (26, 30). Therefore, Type I HBEC appear to be the major target cells for breast carcinogenesis. In contrast, Type II HBEC show basal epithelial phenotypes (i.e., expression of cytokeratin 14 and α-6 integrin), do not express estrogen receptor α, and rarely become immortal after transfection with SV40 large T-antigen (26–28, 31).

For this study, the following criteria were applied to determine the chemotherapeutic and chemopreventive properties of sphingoid bases in transformed highly tumorigenic Type I HBEC (representing breast cancer cells) and Type I (stem cells) and Type II HBEC (normal differentiated cells): (i) a chemotherapeutic agent is expected to inhibit the proliferation and induce apoptosis or terminal differentiation of transformed tumorigenic Type I HBEC, and (ii) a chemopreventive agent is expected to preferentially inhibit the proliferation and induce the differentiation of Type I HBEC to Type II HBEC but does not affect the growth of Type II HBEC. This will decrease the target stem cells for neoplastic transformation.

Materials and Methods

Sphingolipids. Sphinganine (D-erythro-dihydrosphingosine) was obtained from Matreya Co. (cat. no. 1831; State College, PA), and D-erythro-sphingosine was from Sigma-Aldrich Co. (S-6879; St. Louis, MO). Stock solutions of sphingoid bases were dissolved in ethanol at 50 m*M* and stored at 4°C. Working solutions of sphingoid bases were prepared as 1:1 complexes with 1 m*M* bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and stored at 4°C up to 3 weeks. Prior to experiments, sphingoid bases were incubated at 37°C for 8 h and sonicated for 2 h at room temperature to ensure sphingoid bases–BSA complex formation.

Development of HBEC from Reduction Mammoplasty Tissues of Healthy Females and Separation of Type I HBEC and Type II HBEC. Breast tissues of healthy women at 23 and 26 years of age were obtained during reduction mammoplasty at a hospital in Lansing, Michigan and patients' consents were received. The procedure for the development of Type I HBEC and Type II HBEC from reduction mammoplasty tissues has been described previously (26). The initial HBEC cultures developed in a week were stored in liquid nitrogen until use. Type I and Type II HBEC were separated as described previously (26).

Derivation of *In Vitro* Neoplastically Transformed HBEC Lines from Type I HBEC. Previously Kao *et al.* (26) and Kang *et al.* (30) sequentially derived *in vitro* neoplastically transformed HBEC lines (M13SV1, M13SV1R2, and M13SV1R2N1) from Type I HBEC (26, 30; for review, see Ref. 27). In the present study, the transformed highly tumorigenic Type I HBEC line (M13SV1R2N1) was used as a breast cancer cell model.

Culture of Type I HBEC, Transformed Tumorigenic Type I HBEC, and Type II HBEC. The MSU-1 medium with supplements (called "MSU-1+S medium" hereafter) was prepared as described previously (26). During experimental treatments with sphingoid bases and/or cholera toxin, all cell cultures of Type I, Type II, and transformed tumorigenic Type I HEEC were cultured in the same medium, that is, MSU-1+S medium without fetal bovine serum (FBS) or bovine pituitary extract (BPE). Prior to experiments, to optimize the development of cell culture, the Type II HBEC were grown in MSU-1+S medium with 0.4 % BPE, whereas Type I and transformed tumorigenic Type I HBEC were grown in MSU-1+S medium with 5% FBS (26). Cells were grown in 5% CO_2 cell culture incubator at 37°C.

Assessment of Cell Proliferation. Cell cultures at 80% confluence were trypsinized, and cells were seeded at a density of 6×10^4 cells per well in six-well plate and cultured in 2 ml of the MSU-1+S medium with 5% FBS for 24 hrs for transformed tumorigenic Type I HBEC before treatment with sphinganine and sphingosine. For Type I HBEC, cells were cultured in the MSU-1+S medium with 5% FBS for 3 days since Type I HBEC have lower cell plating efficiency and proliferation rate. For Type II HBEC, cells were cultured in the MSU-1+S medium with 5% FBS to facilitate cell attachment for 1 day and then switched to the MSU-1+S medium with 0.4% BPE for 2 days since

Type II HBEC are sensitive to trypsinization and have lower cell plating efficiency after replating in the MSU-1+S medium with 0.4% BPE.

During the treatment period, all cell cultures of Type I, Type II, and transformed tumorigenic Type I HBEC were grown in the MSU-1+S medium without FBS or BPE. Various concentrations of sphinganine and sphingosine were added directly to each dish, and the cells were cultured for up to 6 days. Fresh MSU-1+S medium and treatments were renewed after 3 days. Total nucleic acids concentrations were determined as described previously (11).

Flow Cytometry Analysis of Cell Cycle Progression. Confluent transformed tumorigenic Type I HBEC were trypsinized and seeded at a density of 3.5×10^5 cells/100-mm dish and cultured with 5 ml of the MSU-1+S medium with 5% FBS for 24 hrs before treatment with sphingoid bases. The medium was replaced with MSU-1+S medium, and sphingoid bases were added directly to each dish and the cells cultured for 24 hrs. After treatments, floating cells were collected and combined with 1.5 ml PBS containing 8% FBS (heat inactivated at 50°C for 30 min and filtered with a 0.22-μm filter) in a tube. Attached cells were incubated with trypsin containing 0.1% methylcellulose for 10 min in 5% CO_2 cell culture incubator at 37°C. Trypsinization was terminated by adding 3 ml of PBS with 4% FBS to each dish, and detached cells were transferred to the previous tubes containing floating cells. Then the cells were further processed as described previously (11). The total number of cells analyzed via FACS Vantage Flow Cytometer (Becton Dickinson, San Jose, CA) for each sample was 7000. The percentages of cells in G_0/G_1, S, and G_2/M phases and apoptotic cells (A_0 peaks in the hypodiploid sub G_0/G_1 area to the left of G_0/G_1 diploid peak) were determined as described previously (11).

Assessment of Type I HBEC Differentiation. Type I HBEC, at 5000 cells/60-mm dish with grids, were grown in 4 ml of the MSU-1+S medium with 5% FBS (which supports the growth of Type I HBEC but not Type II HBEC) for 2 days before treatment with sphingoid bases and cholera toxin. Thereafter, cells were cultured in MSU-1+S medium (which supports the growth of both Type I and Type II HBEC) in the presence or absence of sphingoid bases and cholera toxin (C-8052; Sigma-Aldrich). Cholera toxin was used as a positive control (26). These cells were cultured in MSU-1+S medium for 7 days with change of fresh MSU-1+S medium and renewed chemical treatments on day 3 and day 5. At the end of the experiment, the medium was removed, and the cells were washed once with PBS, stained with 1% crystal violet (Sigma-Aldrich). Stained colonies in dishes were air-dried and kept at room temperature.

The colonies of Type I, Type II, and Type I surrounded by Type II HBEC (the cell culture started from single cell plating of pure Type I HBEC) were visually identified and counted under a microscope. To ensure objectivity, the identities of the treatment for each dish were unknown (blinded) to two people who were instructed to score colonies and did not participate in other parts of the differentiation experiment. The same dishes were independently counted by two people using the same criteria.

Immunofluorescence Staining. Type I HBEC, at cell seeding density of 1250 cells per well in a four-well chamber slide, were grown in 1 ml of the MSU-1+S medium with 5% FBS for 2 days, and on the third day cells were cultured in 1 ml of MSU-1+S medium, and the experiment was performed as described in the preceding section. At the end of the experiment, the medium containing floating cells was removed, and the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, rinsed with PBS, and permeabilized with 0.5 % Triton X-100, 2% BSA, and 0.05% NaN_3 in PBS. These cells were sequentially immunostained with a mouse anti-human maspin monoclonal antibody (1:100 dilution, cat. no. 554292; BD Biosciences-Pharmingen, San Diego, CA) and FITC-conjugated sheep anti-mouse IgG (1:200 dilution, cat no. 115-095-166; Jackson ImmunoResearch Laboratories Inc., West Grove, PA). The immunostained cells were photographed using a fluorescence/phase contrast microscope equipped with a digital camera (Diagnostic Instruments, Sterling Heights, MI).

Statistical Analyses. Data for total nucleic acids assay at various concentrations and multiple culture periods were analyzed by two-way analysis of variance (ANOVA). After application of two-way ANOVA, the significance of differences in the means between control and treatment groups at specific culture periods was evaluated by multiple comparisons using the Bonferroni method. Data for total nucleic acids and frequencies of differentiation at various concentrations and a single culture period were analyzed by one-way ANOVA. After application of one-way ANOVA, the significance of differences in the means between control and treatments was evaluated by multiple comparisons using Student-Newman-Keuls method for total nucleic acids data and Dunnett's method for differentiation data. Differences were considered significant at $P < 0.05$.

Results

Sphinganine Inhibited the Growth and Caused Death of Transformed Tumorigenic Type I HBEC More Potently than Sphingosine. Total nucleic acids in control cultures doubled within 1 day and increased 10-fold in 5 days (Fig. 1A and B). Sphinganine (Fig. 1A) caused concentration- and time-dependent decreases in proliferation of transformed tumorigenic Type I HBEC. Data shown in Figure 1A and B indicate that sphinganine inhibited the growth and caused death of transformed tumorigenic Type I HBEC more effectively than sphingosine.

The effects of sphinganine and sphingosine on growth and death of transformed tumorigenic Type I HBEC were further examined at concentrations ranging from 2 to 10 μ*M* for 24 hrs. As shown in Figure 1C and D, sphinganine

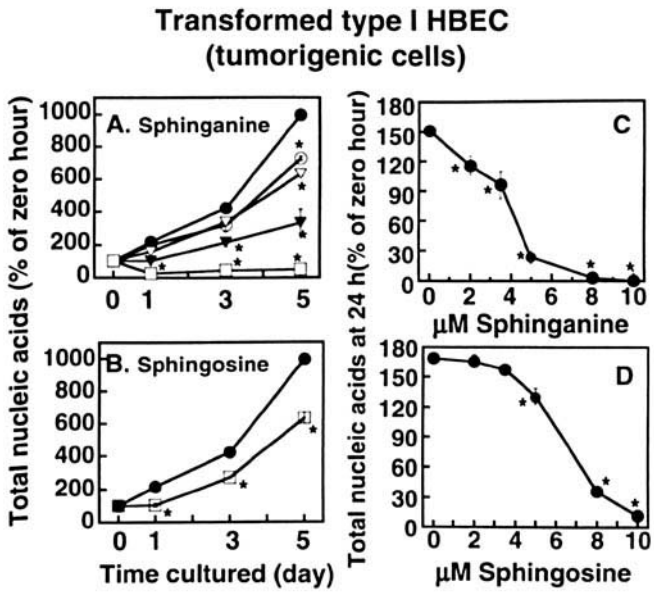

Figure 1. Sphinganine more potently inhibited the growth and caused the death of transformed tumorigenic Type I HBEC than sphingosine. (A and B) Subconfluent cells (6×10^4 cells per well of a six-well plate cultured for 1 day) were cultured with sphinganine at 0 (●), 0.5 (○), 2 (▽), 3.5 (▼), and 5 μ*M* (□) and with sphingosine at 0 (●) and 5 μ*M* (□) for 1, 3, and 5 days. (C and D) Subconfluent cells (6×10^4 cells per well of a six-well plate cultured for 1 day) were cultured with sphinganine and sphingosine at 0, 2, 3.5, 5, 8, and 10 μ*M* for 24 hrs. Total nucleic acids were measured as an index of cell number. Results are expressed as a percentage of the quantity at zero hour. Representative data from one of three independent cell culture experiments are shown (mean ± SEM, $n=3$). Where an error bar is not seen, it lies within the dimensions of the symbol. Means at each culture period with an asterisk (*) are significantly different ($P < 0.05$) from the corresponding control.

inhibited cell growth more significantly than sphingosine at same concentrations in this dose range. The IC_{50} for sphinganine was approximately 4 μ*M* (Fig. 1C), while that for sphingosine was 6.4 μ*M* (Fig. 1D).

Sphinganine Induced Apoptosis of Transformed Tumorigenic Type I HBEC More Potently than Sphingosine. The effects of sphingoid bases on apoptosis of transformed tumorigenic Type I HBEC were analyzed *via* flow cytometry, and results (mean ± SEM) are shown in Figure 3A and E (Fig. 2 shows representative histograms) using concentrations of sphingoid bases found to be growth inhibitory and cytotoxic in total nucleic acids assays (Fig. 1C and D). About 4.3% of the control cells for sphinganine were in the A_0 apoptotic fraction (Fig. 3A), while sphinganine at 3.5, 5, and 8 μ*M* increased the percentage of apoptotic cells to 7.9%, 8.6%, and 10.9%, respectively (Fig. 3A). About 2.5% of the control cells for sphingosine were in the A_0 apoptotic fraction (Fig. 3E). Sphingosine at 5, 8, and 10 μ*M* increased the percentage of apoptotic cells to 3.4%, 4.3%, and 7.8%, respectively (Fig. 3E).

Sphingoid Bases Arrested the Cell Cycle at G_2/M with Reductions in S and/or G_0/G_1 Phases. The effects of sphingoid bases on the cell cycle distribution of

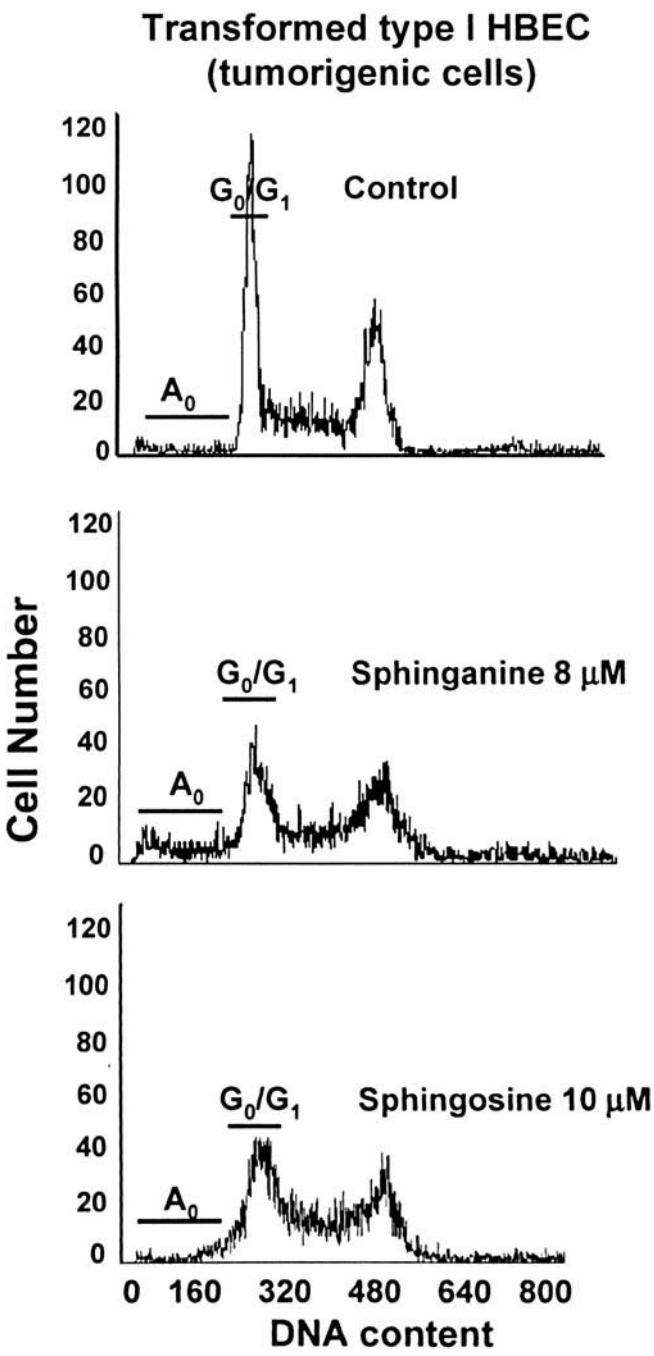

Figure 2. Sphingoid bases increased the number of A_0 (sub-G_0/G_1) cells, indicative of apoptosis, and arrested cell cycle at G_2/M phase in transformed tumorigenic Type I HBEC as shown by representative histograms. Subconfluent cells (3.5×10^5 cells per 100-mm dish cultured for 1 day) were cultured in the absence (control) or presence of sphinganine at 8 μ*M* and sphingosine at 10 μ*M* for 24 hrs and DNA were stained with propidium iodide and were determined *via* flow cytometric analysis.

transformed tumorigenic Type I HBEC were examined using flow cytometry and results (mean ± SEM) are shown in Figure 3B, C, D, F, G, and H (Fig. 2 shows representative histograms). Treatment of sphinganine at 8 μ*M* or sphingosine at 8 and 10 μ*M* for 24 h increased the percentages of cells in G_2/M (Fig. 3D and H) and decreased

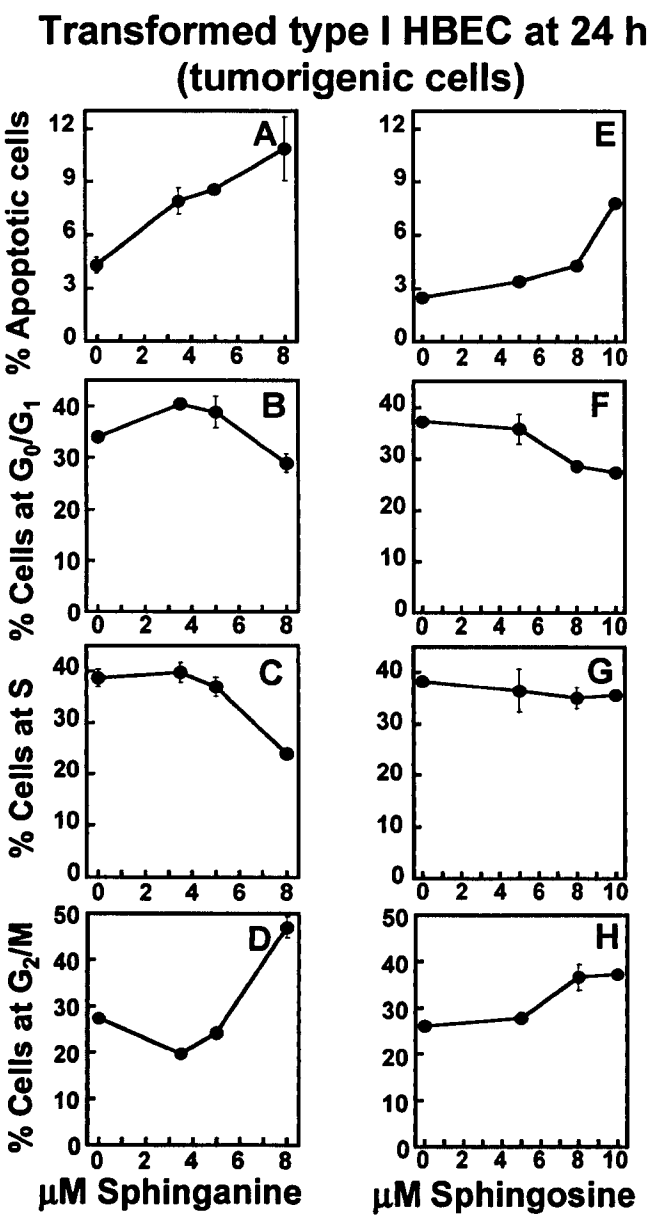

Figure 3. Sphinganine more potently induced apoptosis than sphingosine, while both sphingoid bases at high concentrations (8–10 μ*M*) arrested cell cycle at G_2/M phase with reductions in S and G_0/G_1 phases in transformed tumorigenic Type I HBEC. Subconfluent cells (3.5×10^5 cells per 100-mm dish cultured for 1 day) were cultured with sphinganine at 0, 3.5, 5, and 8 μ*M* and with sphingosine at 0, 5, 8, and 10 μ*M* for 24 hrs and DNA were stained with propidium iodide. The cells in A_0 (sub-G_0/G_1 area), indicative of apoptosis, and in G_0/G_1, S, and G_2/M phases were determined *via* flow cytometric analysis and calculated using FCS Express 2.0 and Mod fit cell cycle analysis software as described in Materials and Methods. Where an error bar is not seen, it lies within the dimensions of the symbol. Representative data from one of two independent experiments are shown (mean ± SEM, $n = 2$, except $n = 6$ for control cells for sphingosine.

the percentages of cells at S (Fig. 3C) and G_0/G_1 (Fig. 3B and F) phases compared to the corresponding control, suggesting that sphingoid bases at these concentrations arrest the cell cycle at G_2/M. Interestingly, sphingosine at both 8 and 10 μ*M* caused similar degrees of accumulations of cells in G_2/M (Fig. 3H), although 10 μ*M* was more potent than 8 μ*M* in inducing apoptosis (Fig. 3E), indicating no linear correlation between degree of apoptosis and accumulation of cells at G_2/M. The observation was similar in repeated independent experiments.

Sphinganine Inhibited the Growth and Caused Death of Type I HBEC More Strongly than Sphingosine. Total nucleic acids in control cultures of Type I HBEC increased more than 3-fold in 6 days (Fig. 4A and B). The growth rate of Type I HBEC (Fig. 4A and B) is much slower than that of transformed tumorigenic Type I HBEC (Fig. 1A and B). As shown in Figure 4A, sphinganine caused concentration- and time-dependent decreases in proliferation of Type I HBEC. At the same concentration of 8 μ*M*, sphinganine (Fig. 4A) inhibited the growth and caused death of Type I HBEC more strongly than sphingosine (Fig. 4B).

Type II HBEC Were Less Sensitive to Growth-Inhibitory Effects of Sphingoid Bases than Transformed Tumorigenic Type I HBEC and Type I HBEC. Total nucleic acids in control cultures of Type II HBEC (normal differentiated human breast epithelial cells) increased more than 4-fold in 6 days (Fig. 4C and D). The growth rate of Type II HBEC (Fig. 4C and D) was also slower than that of transformed tumorigenic Type I HBEC (Fig. 1A and B) but slightly faster than that of Type I HBEC (Fig. 4A and B). As shown in Figure 4C and D, Type II HBEC were less sensitive to growth-inhibitory effects of sphinganine and sphingosine than transformed tumorigenic Type I HBEC (Fig. 1A and B) and Type I HBEC (Fig. 4A and B).

Sphinganine and Sphingosine Induced Differentiation of Type I HBEC to Type II HBEC. Type I HBEC were cultured with sphinganine and sphingosine at non–growth-inhibitory concentrations (0.05, 0.1, and 0.5 μ*M*) for 7 days, and the ability of Type I HBEC to differentiate to Type II HBEC was evaluated by counting morphologically distinguishable colonies of Type I, Type II, and Type I surrounded by Type II HBEC (Table 1 and Fig. 5).

Type I HBEC in vehicle control (treated with 0.1% [v/v] of 1 m*M* BSA and served as the control for sphingoid bases-treated cells) had a 5.4% differentiation rate (frequency of colonies containing Type II HBEC). Control Type I HBEC (not treated with any chemical and served as the control for cholera toxin-treated cells) usually had low colony-forming efficiency and showed a 6.2% differentiation rate (Table 1).

The effects of sphingoid bases were similar within the range of sphingoid bases concentrations tested (0.05, 0.1, and 0.5 μ*M*). Sphinganine at 0.05, 0.1, and 0.5 μ*M* increased the differentiation rate to 12.1%, 13.1%, and 12.4%, respectively. Sphingosine at 0.05, 0.1, and 0.5 μ*M* also increased the differentiation rate to 10.9%, 13.0%, and 9.7%, respectively. Cholera toxin, which is known to induce differentiation of Type I HBEC to Type II HBEC (26), was used as a positive control. The treatment with cholera toxin at 0.1 and 1 ng/ml

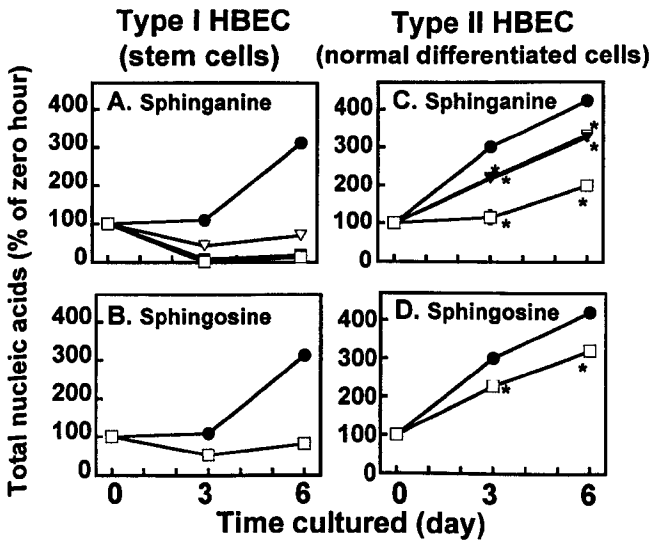

Figure 4. Effects of sphinganine and sphingosine on growth and death of Type I HBEC and Type II HBEC. Subconfluent cells (6×10^4 cells per well of a six-well plate cultured for 3 days) were cultured with sphinganine at 0 (●), 3.5 (▽), 5 (▼), and 8 (□) μ*M* and with sphingosine at 0 (●) and 8 (□) μ*M* for 3 and 6 days. Total nucleic acids were measured as an index of cell number. Results are expressed as a percentage of the quantity at zero hour. Representative data from one of the two independent experiments are shown (mean ± SEM [A and B]: $n=2$, except $n=5$ for 0 days; [C and D]: $n=3$, except $n=6$ for 0 days). Means at each culture period with an asterisk (*) are significantly different ($P < 0.05$) from the corresponding control. Where an error bar is not seen, it lies within the dimensions of the symbol.

increased the frequency of Type II HBEC–containing colonies to 24.4% and 17.7%, respectively (Table 1 and Fig. 5). The differentiation rate increased by sphinganine (0.05, 0.1, and 0.5 μ*M*) and sphingosine (0.05 and 0.1 μ*M*) and Cholera toxin (0.1 and 1 ng/ml) were significantly different from the corresponding control (Table 1; $P < 0.05$).

Sphinganine Increased the Expression of a Tumor Suppressor Protein, Maspin, during Induction of Differentiation of Type I HBEC to Type II HBEC. Type I HBEC were cultured with sphinganine at 0.05 and 0.5 μ*M* for 7 days, and the expression of a tumor suppressor protein, maspin (a protease inhibitor), was determined using immunofluorescence staining. As shown in Figure 6, maspin expression is not detected in Type I HBEC colonies, whereas Type II HBEC–containing colonies show maspin expression.

Discussion

The present study is the first to evaluate the potential of sphinganine and sphingosine as human breast chemotherapeutic and chemopreventive agents in various breast epithelial cell types for relevant comparison (Type I HBEC as stem cells, Type II HBEC as normal differentiated epithelial cells, and transformed tumorigenic Type I HBEC as cancer cells; for review, see Ref. 27). In this study, both sphingosine and sphinganine preferentially inhibited the growth and caused the death of Type I HBEC and transformed tumorigenic Type I HBEC than Type II HBEC. Sphingoid bases at concentrations (sphinganine at 3.5 to 5 μ*M* and sphingosine at 8 μ*M*) that were growth inhibitory and cytotoxic for Type I HBEC (stem cells) and transformed tumorigenic Type I HBEC showed only minor growth-inhibitory effects for Type II HBEC (normal differentiated cells). Thus, our results provide new evidence for chemotherapeutic effect of sphingoid bases. This is consistent with those of Sweeney *et al.* (12), who reported that sphingosine induced apoptosis in SV40-transformed rat mesangial cells but not in normal rat mesangial cells. Recently, Crawford *et al.* (32) reported that novel ceramide analogues, 5R-OH-3E-C8-ceramide, adamantyl-ceramide, and benzene-C_4-ceramide, showed higher cytotoxicity in breast tumor cell lines

Table 1. Sphingoid Bases Induce Differentiation of Type I HBEC to Type II HBEC

Treatments[a]	Total[d]	[II+(I+II)][e]	%[II+(I+II)][f]
Vehicle control (0.1% BSA)[b]	117.0 ± 5.69	6.3 ± 1.45	5.4 ± 1.02
Sphinganine 0.05 μ*M**	104.7 ± 1.45	12.7 ± 0.88	12.1 ± 0.94*
Sphinganine 0.1 μ*M**	125.7 ± 11.26	16.7 ± 2.60	13.1 ± 1.22*
Sphinganine 0.5 μ*M**	137.3 ± 5.84	17.0 ± 2.00	12.4 ± 1.31*
Sphingosine 0.05 μ*M**	101.3 ± 6.01	11.0 ± 0.58	10.9 ± 0.49*
Sphingosine 0.1 μ*M**	59.3 ± 8.09	8.0 ± 2.08	13.0 ± 2.05*
Sphingosine 0.5 μ*M*	71.3 ± 1.21	7.0 ± 2.08	9.7 ± 2.82
Control (nothing added)[c]	32.7 ± 2.91	2.0 ± 0.00	6.2 ± 0.54
Cholera toxin 0.1 ng/ml*	33.0 ± 4.16	7.7 ± 0.88	24.4 ± 4.90*
Cholera toxin 1 ng/ml*	31.7 ± 2.33	5.7 ± 0.88	17.7 ± 1.71*

[a] Type I HBEC were seeded at 5000 cells/60-mm dishes with grids in triplicate and grown for 2 days in the MSU-1+S medium with 5% FBS and then cultured with various chemicals in the MSU-1+S medium for 7 days. Fresh medium with and without chemicals were renewed on days 3 and 5. Data from one of two independent cell culture experiment are shown (mean ± SEM, $n=3$).
[b] Vehicle control (0.1% BSA) is used for sphingoid bases treated cells.
[c] Control (nothing added) is used for cholera toxin–treated cells.
[d] Total = sum of number of colonies of Type I, Type II, and Type I surrounded by Type II HBEC.
[e] [II+(I+II)] = sum of Type II colonies and Type I colonies surrounded by Type II HBEC.
[f] %[II+(I+II)] = [(number of Type II colonies + Type I colonies surrounded by Type II HBEC)/total number of colonies] ×100. % Means with an asterisk (*) are significantly different ($P < 0.05$) from the corresponding control.

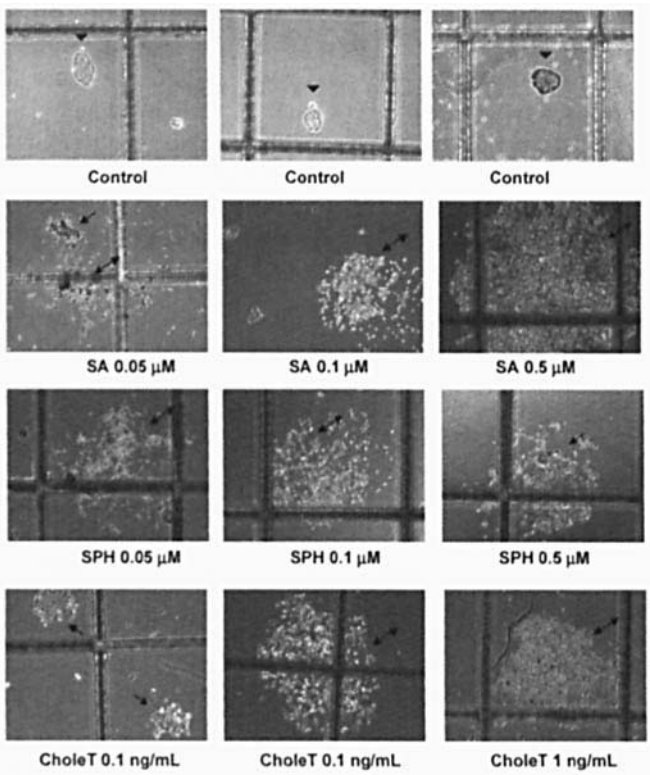

Figure 5. Sphinganine- and sphingosine-induced differentiation of Type I HBEC to Type II HBEC (representative photographs at magnification ×10 plus ×4). Type I HBEC were seeded at 5000 cells per 60-mm dish with or without grids and grown for 2 days and then cultured with sphinganine (SA) and sphingosine (SPH) at 0, 0.05, 0.1, and 0.5 μ*M* and cholera toxin (CholeT) at 0.1 and 1 ng/ml for 7 days. Fresh medium with and without chemicals were renewed on days 3 and 5. The number of colonies of Type I (indicated with ▾), Type II (↔), and Type I surrounded by Type II (→) HBEC were quantitated after 7-day treatments. Cells shown in purple color were photographed after staining with crystal violet. Representative data from one of two independent experiments are shown. Color version of this figure is available online.

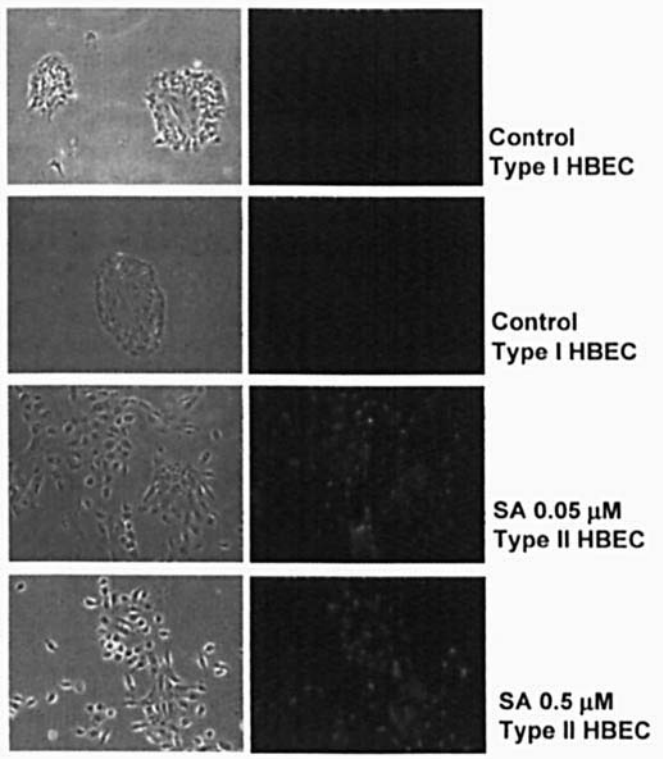

Figure 6. Sphinganine induced the expression of a tumor suppressor protein maspin during induction of differentiation of Type I HBEC to Type II HBEC (photographs). Type I HBEC were seeded at 1250 cells per well in a four-well chamber and grown for 2 days and then cultured with sphinganine (SA) at 0, 0.05, and 0.5 μ*M* for 7 days. Fresh medium with and without chemicals were renewed on days 3 and 5. On day 7, cells were immunostained with an antibody against maspin. Same colonies were observed under UV fluorescence (right panel) or phase contrast (left panel) at magnification ×10 plus ×20. Representative data from one of two independent cell culture experiments are shown. Color version of this figure is available online.

(SKBr3 cells, MCF-7/Adr cells) compared to the normal breast epithelial cells.

The present study demonstrated that sphinganine induces apoptosis of transformed tumorigenic Type I HBEC more potently than sphingosine. This result is consistent with our previous findings in human colon cancer cells (11). The reason for the greater apoptotic potency of sphinganine than sphingosine is unclear. It could be related to relative uptake and sensitivity of the molecular targets of the sphingoid bases and/or activities of the enzymes responsible for sphingoid base metabolism (22, 23; for review, see Refs. 24, 25). Recently Sugawara *et al.* (33) reported that the efflux of sphingadienine of plant origin, but not sphingosine of mammalian origin, was affected by p-glycoprotein. It is also noted that many drugs currently in use for cancer chemotherapy selectively kill target cancer cells by inducing apoptosis. This anticarcinogenic efficacy of many chemotherapeutic agents is associated with sphingolipid metabolism. For example, the chemotherapeutic effect of tamoxifen (34–37) is reduced when generation of acylated-sphingoid base is impaired but is enhanced when the degradation of acylated-sphingoid base is blocked (for review, see Ref. 38).

Our results showed that sphingoid bases at non–growth inhibitory concentrations (0.05–0.5 μ*M*) significantly induced differentiation of Type I HBEC to Type II HBEC. Thus, sphingoid bases might function as a breast cancer chemopreventive agent by reducing the number of breast epithelial stem cells from which breast cancer cells arise (39). The mechanism by which sphingoid bases induced differentiation of Type I HBEC remains unknown. Ceramide was first recognized as a differentiating agent for HL-60 human leukemia cells (40–42), while ceramide-induced differentiation has been studied mostly in neuronal cells and keratinocytes (43; for review, see Refs. 44, 45). Exogenous ceramide mimicked vitamin D_3 (40) or TNFα-induced monocytic differentiation of HL-60 cells by regulating *c-myc* expression (41, 42). Hui *et al.* (46) demonstrated that sphinganine facilitated the retinoic acid–induced differentiation of HL-60 cells. Sphingoid bases might increase cellular cAMP to induce the differentiation since cholera toxin (used as a positive control in the current study), a well-

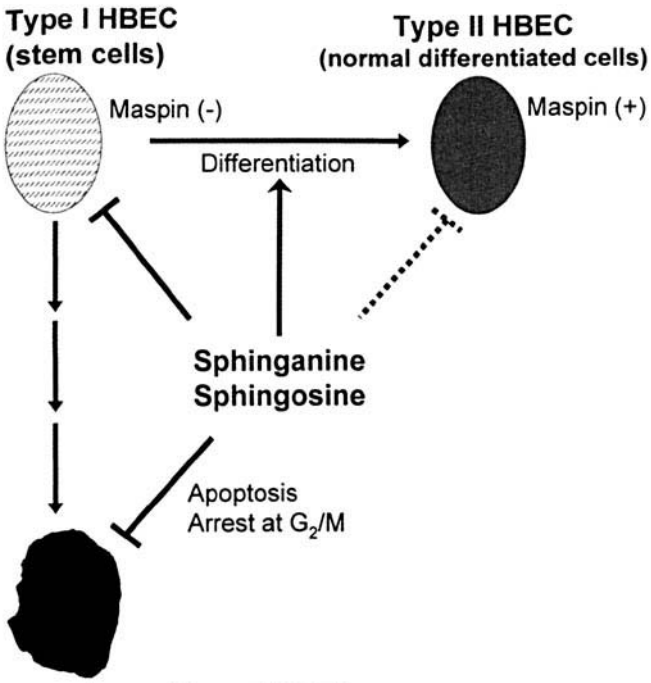

Figure 7. Diagrammatic illustration of chemopreventive properties of sphinganine and sphingosine in Type I HBEC and chemotherapeutic effects of sphinganine and sphingosine against transformed tumorigenic Type I HBEC. The sphingoid bases preferentially inhibit the growth and induced cell death of Type I HBEC and transformed tumorigenic Type I HBEC and induce the differentiation of Type I HBEC to Type II HBEC.

known inducer of cAMP, increased the differentiation of Type I HBEC to Type II HBEC (26).

We demonstrated the detection of maspin expression during sphinganine-induced differentiation of Type I HBEC to Type II HBEC. The expression of maspin in Type II but not in Type I HBEC was demonstrated by immunostaining and Western blotting (47) as well as cDNA microarray (48). The differentiation of Type I to Type II HBEC may involve a wholesale switch in gene expression including maspin (26, 27, 48). Maspin (mammary serpin), a serine protease inhibitor and a tumor suppressor, was originally isolated from normal breast epithelial cells, and its expression was decreased during tumor progression (49–51). The nonexpression of maspin in breast cancer cells appears to be the continuation of a parental breast epithelial stem cell phenotype (47). Our results suggest that maspin expression can serve as a biomarker together with other biomarkers we previously reported (i.e., Oct-4, α6-integrin, connexin 26, connexin 43, cytokeratin-14, and so on) (27, 52) for efficacy of potential breast cancer chemopreventive agents.

In summary, the results of this comparative study to evaluate the chemotherapeutic and chemopreventive potential of sphinganine and sphingosine using various relevant human breast epithelial cell types (stem cells, normal differentiated cells, and tumorigenic cells) have provided evidence that sphingoid bases may function as chemotherapeutic and chemopreventive agents against human breast cancer as diagrammatically shown in Figure 7. The validation and application of sphingoid bases should be explored in future studies, such as the effect at different developmental stages for chemoprevention and the efficacy of systematic vs. intratumor delivery of sphingoid bases for chemotherapeutic effect in conjunction with other anticancer agents.

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