Chemotherapeutic and chemopreventive roles of sphingolipids in human breast cancer

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ABSTRACT

CHEMOTHERAPEUTIC AND CHEMOPREVENTIVE ROLES OF SPHINGOLIPIDS IN HUMAN BREAST CANCER

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Complex sphingolipids are significant constituents of food (Ahn and Schroeder, 1998) and are digested and absorbed as the bioactive metabolites, sphingosine and ceramide (Schmelz *et al.*, 1994). Sphingosine and the cell-permeable, ceramide-analog C_2-ceramide have been shown to inhibit proliferation and cause death of estrogen receptor-negative, MDA-MB-231 human breast cancer cells (Zhang and Schroeder, 1998) and induce differentiation of HL-60 leukemia cells (Okazaki *et al.*, 1990). The objectives of this study were to assess the chemotherapeutic potential of sphingosine and C_2-ceramide by comparing their effects on proliferation and death of breast cancer cells to conventional normal human breast epithelial cells (HBEC) which have a basal cell phenotype (Type II HBEC) and to assess the chemopreventive potential of these sphingolipids by examining their effects on proliferation, differentiation and apoptosis of HBEC which have stem cell characteristics and are susceptible to carcinogenesis (Type I HBEC). The results show that both sphingosine and C_2-ceramide inhibit proliferation and cause death of tumorigenic breast cells (*in vitro* neoplastically transformed Type I HBEC and MCF-7 and MDA-MB-231 breast cancer cells). The inhibition of tumorigenic cell growth by sphingosine was accompanied by dephosphorylation of retinoblastoma protein and inhibition of telomerase activity. Death of transformed Type I HBEC and MDA-MB-231 cells caused by sphingosine and C_2-ceramide had characteristics of apoptosis (*i.e.* morphological changes and formation of a DNA ladder on agarose gel

electrophoresis). The sensitivities of the tumorigenic breast cell lines to C_2-ceramide are similar to that of the Type II HBEC. However, the tumorigenic breast cells are more sensitive to inhibition of proliferation by sphingosine than Type II HBEC. This suggests that sphingosine is a potential chemotherapeutic agent for breast cancer; whereas, C_2-ceramide might be toxic for normal human breast tissue at concentrations that are anti-proliferative for cancer cells. The sensitivities of Type I HBEC to growth inhibition by sphingosine and C_2-ceramide were similar to that of tumorigenic breast cells. C_2-ceramide appeared to cause death of Type I HBEC by inducing apoptosis. At non-cytotoxic concentrations (1-3 μM), sphingosine induced differentiation of Type I HBEC; whereas, C_2-ceramide did not affect differentiation. Therefore, sphingosine might also function as a chemopreventive agent by inducing the differentiation of Type I HBEC with stem cell characteristics and, thereby, reducing the targets for neoplastic transformation.

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I. INTRODUCTION

Breast cancer is the most common cancer and the second leading cause of cancer-related deaths in women in the United States (Cancer Facts and Figures, 1998). The disease arises as a result of the accumulation of mutations of critical genes that regulate cell proliferation, differentiation and apoptosis in breast cells (Russo *et al.*, 1990). The majority of breast cancers are adenocarcinomas originating from epithelial cells (Osteen *et al.*, 1986). Terminal end buds, which contain highly proliferating mammary epithelial stem cells, are considered to be the target of mammary neoplastic transformation (Russo and Russo, 1987). Chemotherapeutic agents are being investigated with the goal of treating this disease without side effects or the development of drug resistance. Recent research also has focused on identifying specific dietary components that may prevent the development of breast cancer (Love, 1994).

Complex sphingolipids are significant constituents of food (Vesper, *et al.*, 1999, Ahn and Schroeder, 1998) and are digested and absorbed as the bioactive metabolites ceramide and sphingosine (Schemelz *et al.*, 1994). Ceramide and sphingosine regulate cell behavior including cell proliferation, differentiation and apoptosis (Merrill *et al.*, 1995). Ceramide may mediate the effects of ionizing radiation and the breast cancer chemotherapeutic agents vincristine and doxorubicinn which have been shown to cause cellular accumulation of ceramide (Hannun, 1997). Since ceramide can be deacylated by ceramidase to form sphingosine, some cellular effects originally attributed to ceramide might be mediated by sphingosine (Ohta *et al.*, 1994). Because of their abilities to inhibit proliferation and induce apoptosis in breast cancer cell lines (Gill *et al.*, 1997; Cai *et al.*, 1997; Zhang and Schroeder, 1998), ceramide and sphingosine may be useful agents to treat breast cancer. However, no

study has examined the effects of ceramide and sphingosine on the proliferation of normal breast epithelial cells or evaluated the chemopreventive potential of these sphingolipids.

Recently, two types of morphologically distinguishable normal human breast epithelial cells (HBEC) were derived from reduction mammoplasty (Kao *et al.*, 1995). Type I HBEC express estrogen receptors (Kang *et al.*, 1997) and have stem cell characteristics (*i.e.* deficiency in gap junctional intercellular communication, ability to differentiate to Type II HBEC and to form budding/ductal structures on Matrigel) (Kao *et al.*, 1995; Sun *et al.*, 1999). Significantly, Type I HBEC are susceptible to neoplastic transformation and SV40 large T-antigen transformed Type I HBEC were capable of anchorage independent growth and were more susceptible to telomerase activation and immortalization (Kao *et al.*, 1995; Sun *et al.*, 1999). Therefore, Type I HBEC appear to be the target cells for neoplastic transformation. In contrast, Type II HBEC express basal epithelial cell markers (*i.e.* α-6 integrin and cytokeratin-14) and rarely become immortal after SV40 transformation.

The specific aims of the proposed studies were to use the unique HBEC culture system as well as MCF-7 and MDA-MB-231 breast cancer cells to determine if ceramide and sphingosine have: 1) Chemopreventive activities-Are ceramide and sphingosine capable of inhibiting the proliferation and/or inducing the differentiation of Type I HBEC, thereby reducing the targets for breast carcinogenesis?; and 2) Chemotheraputic activities-Do sphingosine and ceramide inhibit proliferation and induce apoptosis of tumorigenic breast cells more potently than for normal Type II HBEC?

II. LITERATURE REVIEW

A. Breast cancer

1. Breast cancer epidemiology-Breast cancer is the most common cancer and the second leading cause of cancer-related deaths in women in the United States. About 178,700 new invasive cases were estimated to occur in 1998 in the United States and the incidence of breast cancer is approximately 110 cases per 100,000 women (Cancer Facts and Figures, 1998). Although the mortality rate has stabilized in recent years mainly due to earlier detection and improved treatment, there were still an estimated 43,500 deaths during 1998.

2. Breast cancer etiology-Breast cancer arises as a result of the accumulation of mutations of critical genes that regulate cell proliferation, differentiation and death in breast cells (Osteen *et al.*, 1986). The majority of breast cancers are adenocarcinomas originating from epithelial cells. Terminal end buds, which contain highly proliferating mammary epithelial stem cells, are considered to be the target of mammary neoplastic transformation (Russo and Russo, 1987). Many risk factors for breast cancer in females have been documented (Marshall *et al.*, 1993). The most significant ones include old age, early menarche, late first full-term pregnancy, late menopause, obesity after menopause, ovariectomy, history of fibrocystic disease and history of primary cancer in ovary or endometrium, family history of premenopausal bilateral breast cancer and place of birth (North America, Europe >Asia, Africa).

3. Breast cancer and diet-The diet is a complex mixture containing both pro-carcinogenic and anti-carcinogenic agents. The correlation of higher breast cancer incidence with early menarche and taller stature may be reflective of the influence of nutrition during childhood and adolescence (Pollner, 1993). Animal studies show that caloric restriction

reduces breast cancer incidence (Klurfeld *et al.*, 1991). Obese postmenopausal women have a higher risk of breast cancer (Cleary and Maihle, 1997) and overweight breast cancer patients have a poorer prognosis (Bastarrachea *et al.*, 1994). High alcohol consumption increases the risk of breast cancer (Davis *et al.*, 1993) while dietary carotenoids (Verhoeven *et al.*, 1997) and vitamins A (Sankaranarayanan and Mathew, 1996), C (Verhoeven *et al.*, 1997) and E (Kimmick *et al.*, 1997) may help to prevent breast cancer mainly by acting as antioxidants. Blood 1,25-dihydroxyvitamin D_3 levels are associated with low risk of breast cancer (Janowsky *et al.*, 1997), probably through regulating proliferation and differentiation of breast cells (Reichel *et al.*, 1989).

Epidemiology studies suggest a reduced risk of breast cancer in women who consume fermented milk products (Veer *et al.*, 1989) and *in vitro* studies show antiproliferative activity of fermented milk in MCF-7 breast cancer cells (Biffi *et al.*, 1997). Milk fat components including conjugated linoleic acid, butyric acid, ether lipids, and sphingomyelin show anticarcinogenic effects in animal experiments (Parodi, 1997).

Soy intake was found to be inversely correlated with the risk of breast cancer among 142,857 women in Japan over a period of 17 years (Messina *et al.*, 1994) and diets containing soybeans reduced mammary tumor occurrence induced by irradiation (Troll *et al.*, 1980) and the chemical carcinogen, N-methyl-N-nitrosourea (MNU) in animal models (Barnes *et al.*, 1988). Phytoestrogens present in soybeans appear to help prevent mammary cancer (Messina *et al.*, 1997).

4. Role of stem cell differentiation in breast cancer prevention-One important histologic feature of malignancy is anaplasia (*ie.* a loss of differentiation). As a result, cancer has been described as a disease of differentiation (Markert, 1968) or oncogeny as blocked or

partially blocked ontogeny (Potter, 1978 and 1987). Stem cells are undifferentiated cells that are capable of proliferation and self-maintenance, and which produce a large number of differentiated progeny cells (Loeffler, 1997). The relatively undifferentiated nature of tumor cells could be due to the de-differentiation of differentiated cells or blocked differentiation in stem cells which give rise to cancer cells (Varmur and Weinberg, 1993). As mentioned before, early full-term pregnancy decreases risk for breast cancer. This might be related to stem cell multiplication that occurs beginning at the time of puperty and during each ovarian cycle until, but not after, the first pregnancy (Cairns, 1975). Alternatively, pregnancy may induce full differentiation of the mammary gland (Russo *et al.*, 1990), thus decreasing the susceptibility for carcinogenesis by reducing the number of stem cells. Similarly, dietary components that reduce the incidence of breast cancer may act by reducing the number of stem cells.

5. Breast cancer chemotherapy and apoptosis-Apoptosis is a type of programmed cell death with distinctive morphological and biochemical changes which allows deletion of unwanted cells from an organism (Vaux *et al.*, 1996). In apoptosis, the nuclear chromatin is condensed and aggregates under the nuclear membrane. Then, activation of endonucleases causes fragmentation of DNA into multiples of 200 base pair nucleosome-sized pieces. Cells shrink, exhibit cytoplasmic budding, and fragment into membrance-bound vesicles of cytosol and organelles termed apoptotic bodies. Normally, apoptotic bodies are phagocytosed and degraded without eliciting an inflammatory response in the surrounding tissue (Walker *et al.*, 1988). The occurrence of apoptosis can be determined by the appearance of morphologic alterations and endonucleosomal DNA fragments.

Apoptosis-inducing agents which selectively kill cancer cells and/or have less effects

on neighboring normal cells would be ideal chemotheraputic agents. In fact, many chemotherapeutic agents cause cancer cell death mainly by inducing apoptosis (Hannun, 1997). Different types of cells vary in their susceptibility to apoptotic induction; thus, treatments may induce apoptosis in tumor cells while arresting the cell cycle in normal cell counterparts (Fisher, 1994). The apoptosis pathway may be disrupted in tumor cells, which leads to both a survival advantage and resistance to treatment (Fisher, 1994). Alternatively, improper stimulation of proliferation may lead to apoptosis since proto-oncogenes such as c-*myc* (Green, 1997) as well as c-*fos* and c-*jun* (Preston *et al.*, 1996) which stimulate cell proliferation can also induce apoptosis.

6. Telomerase activity, a biomarker of cell cycle progression and differentiation- Telomeres are repetitive TTAGGG sequences at the ends of eukaryotic chromosomes that shorten by 50-200 base pairs after each cell division because of the incomplete replication of the 5' ends of DNA molecules (Shay *et al.*, 1993). Telomeres are required for proper chromosome segregation during mitosis by preventing nuclease degradation and end-to-end fusion of chromosomes during replication (Kirk *et al.*, 1997). Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric DNA, thereby preventing the replication-dependent shortening of DNA. Telomerase activity is detected in 85% to 95% of immortal and tumor cells including breast cancer cells but rarely in normal cells except germ cells and stem cells (Shay *et al.*, 1993, Belair *et al.*, 1997). In human breast cancer, telomerase activity is associated with cell cycle regulatory defects such as overexpression of cyclin D_1 and/or cyclin E (Landberg *et al.*, 1997). Also, absence of telomerase activity was reported in G_2/M-synchronized MCF-7 and MDA-435 breast cancer cells (Zhu *et al.*, 1996). Recently, telomerase also has been used as a biomarker of cell differentiation because of the correlation

between telomerase activity and classification of colorectal carcinomas (Okayasu *et al.*, 1998), esophageal cancer (Asai *et al.*, 1998), prostate cancer (Uemura *et al.*, 1998) and leukemia (Zhang *et al.*, 1996). Telomerase activity also is correlated with tumor aggressiveness and therapeutic effects (Hoos *et al.*, 1998). Inactivation of telomerase activity and differentiation therapy are new therapeutic approaches in prostate cancer (Schalken, 1998). The regulation of telomerase in cancer cells is not clear; however, protein kinase C (PKC) inhibitors such as sphingosine were shown to specifically inhibit telomerase activity in human nasopharyngeal cancer cells (Ku *et al.*, 1997).

B. Sphingosine and ceramide regulate cell behavior

1. Sphingolipid metabolism-Sphingolipids are bioactive molecules with a sphingoid base backbone. Complex sphingolipids, such as sphingomyelin, are major constituents of all eukaryotic and some prokaryotic cells and account for about 20% of plasma membrane lipid (Kolesnick *et al.*, 1991). Sphingomyelin has a sphingoid base backbone primarily composed of sphingosine, an amide-linked fatty acid and a phosphorylcholine polar head group (Figure 1). Upon stimulation with agonists such as 1α, 25-dihydroxyvitamin D_3 (Okazak *et al.*, 1989 and Nikolova *et al.*, 1997), tumor necrosis factor-α, γ-interferon (Kim *et al.*, 1991) and interleukin-1β (Ballou *et al.*, 1992 and Nikolova-Karakashian *et al.*, 1997), sphingomyelin is hydrolyzed to ceramide and/or sphingosine (Nikolova-Karakashian *et al.*, 1997). Both ceramide and sphingosine act as second messengers that mediate diverse cellular behaviors (Figure 2) including cell proliferation, differentiation and apoptosis (Merrill *et al.*, 1997). Because of their ability to inhibit proliferation and induce apoptosis in certain tumor cell lines, sphingolipids may be useful agents in treating various cancers. As components of food, complex sphingolipids are found in relatively high concentration in soybeans, eggs and dairy

products (Vesper *et al.*, 1996, Ahn and Schroeder, 1998). About 90% of complex sphingolipids are digested and absorbed throughout the small intestine to ceramide, sphingosine and other metabolites; however, about 10% reaches the colon (Schemelz *et al.*, 1994). Feeding milk sphingomyelin was shown to suppress the appearance of both the dysplasia lesions and the more advanced malignant colon tumors in rats treated with the colon carcinogen 1,2-dimethylhydrazine (Schemelz *et al.*, 1996). Dietary sphingomyelin can increase serum sphingomyelin in a dose-dependent manner (Imaizumi *et al.*, 1992), which suggests that dietary sphingolipids can also reach other organs via the circulation.

2. Ceramide inhibits cell proliferation, causes differentiation, and induces apoptosis-Cell-permeable ceramide analogues, such as C_2-ceramide and C_6-ceramide can mimic the effects of exogenous stimuli, which supports a role of ceramide in mediating multiple cellular functions triggered by agonists. Among them, the most distinctive actions are inhibition of proliferation and induction of apoptosis (Kolesnick and Kronke, 1998). In addition, ceramide may mediate the effects of ionizing radiation and the breast cancer chemotherapeutic agents vincristine and doxorubicinn which have been shown to cause accumulation of ceramide (Hannun, 1997). Ceramide inhibits the proliferation of normal fibroblasts (Hannun *et al.*, 1994) and induces apoptosis in fibroblasts (Cifone *et al.*, 1994) in a number of other cell lines including human myeloid leukemia cells (Jarvis *et al.*, 1994), human pancreatic cancer cells (Yamada *et al.*, 1997), prostate cancer cells (Herrmann *et al.*, 1997), oligodendrocytes (Larocca *et al.*, 1997) and rat neonatal cardiomyocytes (Bielawska *et al.*, 1997). In HL-60 leukemia cells, C_2-ceramide inhibited cell proliferation at concentrations as low as 1-10 μM and induced cell differentiation (Okazaki *et al.*, 1989). Exogenous C_6-ceramide (15 μM) induced cell cycle arrest at the G_0/G_1 phase in Molt-4 T

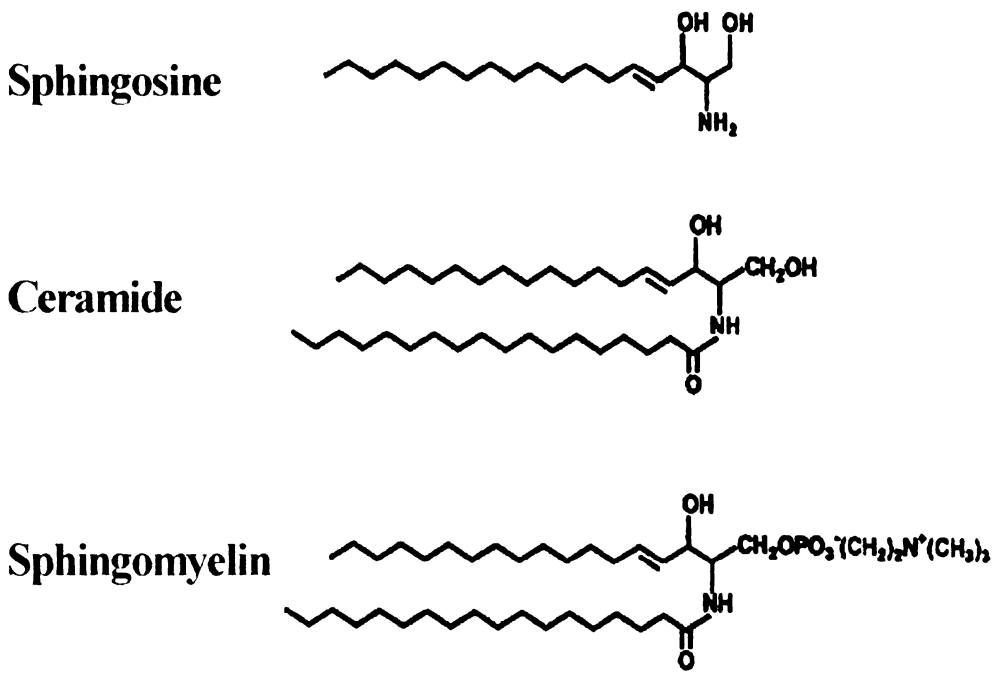


Figure 1. Structures of sphingosine, ceramide and sphingomyelin

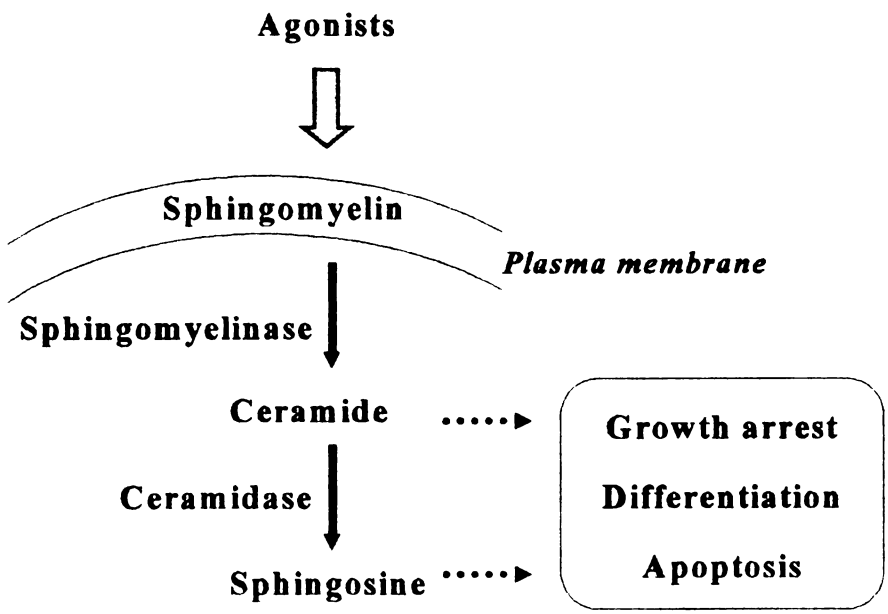


Figure 2. Sphingomyelin turnover pathway.

leukemia cells and Wi 38 human fibroblast cells (Jayadev *et al.*, 1995).

3. Sphingosine inhibits cell proliferation, causes differentiation, and induces apoptosis-Ceramide can be deacylated by ceramidase to form sphingosine. Therefore, some cellular effects originally attributed to ceramide might be mediated by sphingosine (Ohta *et al.*, 1994). One piece of evidence that supports this hypothesis is that exogenously added sphingosine induced apoptosis much earlier than ceramide in human neutrophils (Ohta *et al.*, 1995). Sphingosine induced apoptosis in human myeloid leukemia cells, human prostatic carcinoma cells (Shirahama *et al.*, 1997), and other solid tumor cell lines (Sakakura *et al.*, 1996, Sweeney *et al.*, 1996). An interesting observation is sphingosine induces apoptosis in SV-40 transformed epithelial cells such as HUVECS and rat mesangial cells, but not in their primary culture counterparts (Sweeney *et al.*, 1996), which implies sphingosine may be an excellent candidate as an anti-cancer agent.

Sphingosine inhibited the proliferation of Chinese hamster ovary cells (Merrill, *et al.*, 1989) and human T-lymphocytes (Borchaidt *et al.*, 1994), and caused cell-cycle arrest at G_o/G_1 in HL-60 cells (Chao *et al.*, 1992). Like the induction of apoptosis, the effects of ceramide on cell proliferation and differentiation could also be mediated by sphingosine. In neuroblastma Neuro 2a cells, there was a concomitant, early and sustained increase in the ceramide concentration during retinoic acid-induced differentiation; whereas, supplying either sphingosine or ceramide induced neurite formation and inhibited thymidine incorporation into DNA (Riboni *et al.*, 1995).

The mechanisms whereby sphingosine inhibits tumor cell proliferation and induces apoptosis are not fully understood. Sphingosine inhibits protein kinase C isoenzyme family members and has anti-proliferative properties (Hannun *et al.*, 1986). Sphingosine is also a

potent inhibitor of a mammalian RNA primase *in vitro* (Simbulan *et al.*, 1994) and the suppression of proliferation of human leukemic HL-60 cells was correlated with DNA primase inhibition (Tamiya *et al.*, 1997). Sphingosine (15 μM) induced apoptosis in androgen-independent human prostatic carcinoma DU-145 cells by down-regulation of either *bcl*-2 or *bcl*-X_L gene expression (Sakakure *et al.*, 1996[1,2]). Sphingosine was also shown to down-regulate *c-myc* gene expression and caused retinoblastoma protein (Rb) dephosphorylation (Hannun *et al.*, 1993, Merrill *et al.*, 1991, 1993). In hematopoietic cells, sphingosine-induced dephosphorylation of retinoblastoma protein preceded inhibition of proliferation and cell cycle arrest (Chao *et al.*, 1992).

4. Ceramide and sphingosine have the potential to prevent and treat breast cancer-The potent inhibition of proliferation and induction of differentiation and apoptosis in certain tumor cell lines by ceramide and sphingosine suggests that they could be useful agents in the treatment of cancer. Previous studies showed that ceramide and sphingosine induced cell death in estrogen receptor-negative MDA-MB-231 human breast cancer cells (Zhang and Schroeder, 1998); however, only sphingosine, but not ceramide, caused a DNA ladder in agarose gel electrophoresis and the formation of a pre-G_o/G_1 peak in flow cytometric analysis, both of which are indications of apoptosis. Other studies suggest that ceramide can induce apoptosis in human breast cancer cell lines. C_2-ceramide induced a dose-dependent increase in apoptosis in HS 578T and there was a significant increase in the percentage of cells in the pre-G_1 phase of the cycle in cells treated with as low as 4 μM C_2-ceramide for 24 hours (Gill *et al.*, 1997). C_6-ceramide caused death of MCF-7 cells in a dose-dependent manner at 48 hours by inducing apoptosis (Cai *et al.*, 1997). In the present study, we have investigated the potential of ceramide and sphingosine to prevent and treat human breast

cancer using breast epithelial and cancer cell models.

C. Normal human breast epithelial and cancer cells in culture as experimental models.

Recently, two types of morphologically distinguishable normal human breast epithelial cells (HBEC) were derived from reduction mammoplasty (Kao *et al.*, 1995, Figure 3 and 4). Type I HBEC express luminal epithelial cell markers (*i.e.* epithelial membrane antigen, cytokeratin-18, 19) and have stem cell characteristics (*i.e.* deficiency in gap junctional intercellular communication, ability to differentiate to Type II HBEC and to form budding/ductal structures on Matrigel) (Kao *et al.*, 1995; Sun *et al.*, 1999). Type I HBEC express estrogen receptors (Kang *et al.*, 1997) and are more susceptible to telomerase activation and imortalization after SV40 transformation (Sun *et al.*, 1999). Type I HBEC and these SV40 transformed cells were also capable of anchorage independent growth (Kao *et al.*, 1995). Furthermore, neoplastically transformed Type I HBEC, similar to breast carcinomas, possess many phenotypes of Type I HBEC (*i.e.* expression of epithelial membranc antigen, cytokeratin 18 and estrogen receptors, and deficiency in gap junctional intercellular communication) (Kao *et al.*, 1995; Kang *et al.*, 1997). Therefore, Type I HBEC appear to be the target cells for neoplastic transformation. In contrast, Type II HBEC which express basal epithelial cell markers (α-6 integrin and cytokeratin-14) and gap junction genes (connexin 26 and 43) but not estrogen receptors, did not show anchorage independent growth and rarely became immortal after SV40 transformation (Kao *et al.*, 1995, Sun *et al.*, 1999, Figure 4).

Type I HBEC are an excellent cell model to study the chemopreventive potential of sphingolipids for human breast cancer because they have stem cell characteristics and stem cells appear to be the targets of breast carcinogenesis. In addition, Type II HBEC and

A

B

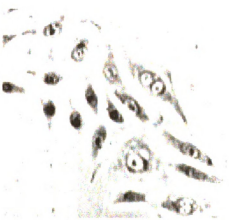


Figure 3. Morphology of two types of normal human breast epithelial cells (HBEC) (photographs)

tumorigenic breast cancer cells can be used to compare the effects of sphingolipids on the proliferation, differentiation and death of normal human breast epithelial cells and breast cancer cells to evaluate their chemotheraputic potential.

We hypothesize that: (1) Sphingosine and ceramide may inhibit proliferation and induce differentiation of Type I HBEC. If so, they may be chemopreventive to human breast cancer by reducing the target cells for neoplastic transformation and inhibiting the proliferation of initiated (precancerous) cells; (2) Sphingosine and ceramide may also inhibit the proliferation or trigger the apoptotic death of the neoplastic transformed Type I HBEC and MCF-7 cells, and thus may be useful as chemotherapeutic drugs for human breast cancer.

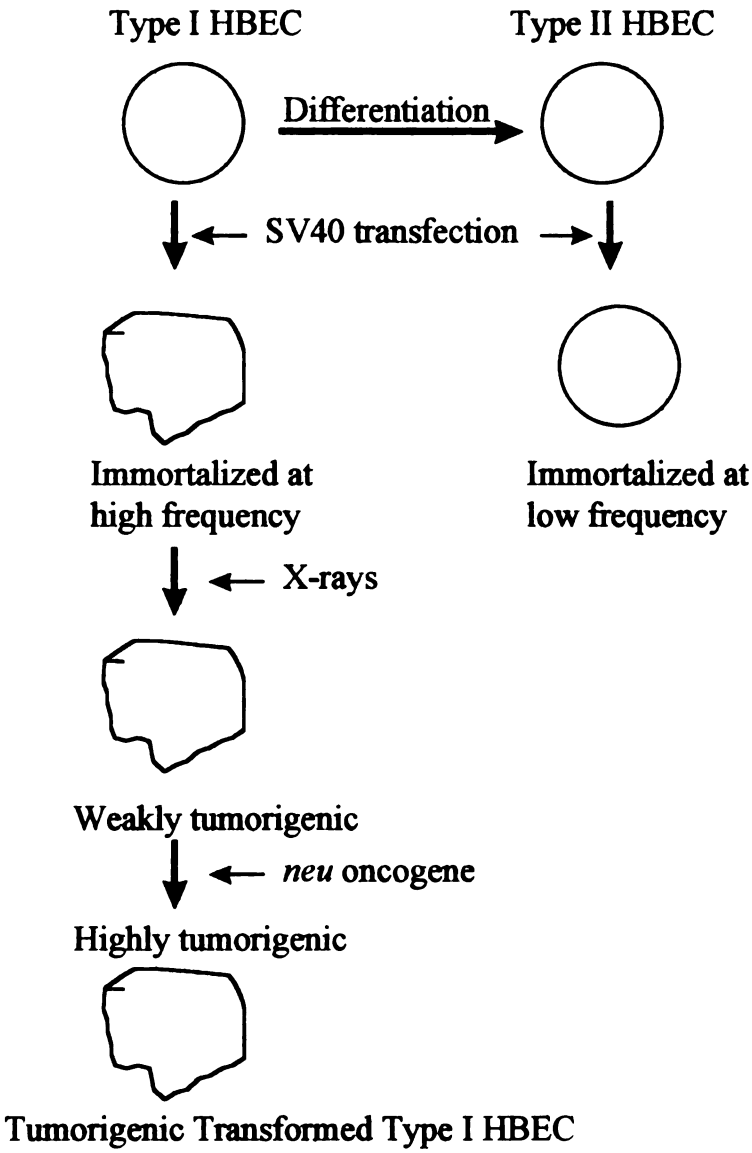


Figure 4. Type I HBEC derived from reduction mammoplasty have the ability to differentiate to Type II HBEC and are susceptible to neoplastic transformation.

III. MATERIALS AND METHODS

Cell culture and sphingolipids treatment-Type I and Type II normal HBEC, an *in vitro* neoplastically transformed Type I HBEC (M13SV1R2N1) (Kang *et al.*, 1998), MCF-7 and MDA-MB-231 breast cancer cells were kindly provided by Dr. C. C. Chang. Type I HBEC were cultured in MSU-1 medium (Kao *et al.*, 1995) with 5% fetal bovine serum (FBS). Type II HBEC were cultured in FBS-free MSU-1 medium supplemented with bovine pituitary extract (0.4 % V/V). A modified Eagle's MEM (D medium) (Chang *et al.*, 1981) with 5 % FBS was used to culture transformed Type I HBEC and breast cancer cell lines. Cells were cultured in incubators at 37°C and supplied with 5% CO_2 and humidified air. Sphingolipids were obtained from Matreya, Inc. Sphingosine was added as a 1:1 complex with bovine serum albumin (BSA) dissolved in phosphate buffered saline (PBS) while C_2-ceramide was dissolved in 70% ethanol.

Assessment of cell proliferation-Cell proliferation was measured by quantitation of total nucleic acid extracted from the cultures (Li *et al.*, 1990). Briefly, cells (6×10^4) were cultured in 6-well dishes in triplicate. The next day, various concentrations (1-10 μM) of C_2-ceramide and sphingosine were added to FBS-free medium. Cells were incubated for 5 days with a change to fresh medium and treatments on day 3. The cells were harvested by rinsing twice with PBS followed by lysis with 1 mL of 0.1 N NaOH. Cell lysates were transfered to 2.2 mL Eppendorf tubes and centrifuged at 14,000 rpm for 2-3 min. The clear lysates were measured for absorbance at 260 nm using a Beckman DU-7400 spectrophotometer.

Assessment of Type I HBEC differentiation-Starting from single cell platings of pure Type I HBEC, the differentiation of Type I HBEC was measured by counting the number of Type II HBEC colonies and colonies of Type I surrounded by Type II HBEC. The

percentage of these colonies among total colonies indicates the differentiation potential of Type I HBEC under different treatments. Briefly, Type I HBEC (5×10^3) were cultured in MSU-1 medium containing 5% FBS in triplicate in 60 mm dishes with grids. On the third day, various concentrations (1-3 μM) of C_2-ceramide and sphingosine were added to FBS-free MSU-1 medium. Cells were incubated for 12 days with change of medium and renewed treatments every 2 days. Cholera toxin (CT) (1 ng/mL) was used as a positive control (Kao et al., 1995). Then, Type I, Type I surrounded by Type II and Type II colonies were visually identified under a microscope and quantitated. The identity of the treatment for each dish was unknown (blind) during counting to ensure objectivity.

Analysis of DNA fragmentation by agarose gel electrophoresis-Cells were trypsinized and centrifuged at 2.2 K rpm for 10 min. Pellets were resuspended in 200 μL PBS and DNA was extracted with 400 μL phenol chloroform followed by centrifugation at 14 K rpm for 5 min. After incubation with 0.2 mg/mL RNase A at 37° C for 1 hr, the extraction was repeated with 400 μL phenol/chloroform to inactivate RNase A. Twenty μL of 3 M NaOAc and 500 μL of 100% ethanol were added to the solution before storing at –20°C overnight. After centrifugation at 14 K rpm (4° C) for 30 min, DNA pellets were washed with 70% ethanol and dried *en vacuo*. The DNA pellets were then dissolved in Tris-EDTA (pH= 8) and the DNA was separated on a 2% agarose gel at 100 V for 1 hr.

SDS-PAGE and Western blotting-Proteins were extracted from cells in 100 mm dishes by treatment with 20% SDS lysis solution containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM antipain, 0.1 μM aprotinin, 0.1 μM sodium orthovanadate, 5 mM sodium fluoride). After sonication via three 10-s pulses with a probe sonicator, the cell lysates were stored at –20°C until use. The

protein amounts were determined using the DC protein assay kit (Bio-Rad Co., Richmond, CA). Proteins were separated on 12.5% SDS polyacrylamide gels and transferred to PVDF membranes at 20 V for 16 hr. After blocking with 5% dried skim milk in PBS containing 0.1% Tween 20, the membrane were exposed to an anti-pRb monoclonal antibody (Oncogene Science, NY). This was then followed by incubation with horseradish peroxidase-conjugated secondary antibody and detected with the ECL chemiluminescent detection reagent (Amersham Co., Arlington Heights, IL). X-ray film was exposed to membranes for 15 s to 1 min (Kang *et al.*, 1996).

Flow cytometric measurement and cell cycle analysis-A quantitative measurement of cell cycle distribution was obtained by flow cytometric analysis of DNA content-cell number frequency histograms, as described by Fraker *et al.* (1995). Briefly, cells were collected after 2 day treatment by trypsinization followed by centrifuging at 1200 rpm for 5 min. Then the cells were resuspended in 5 mL of PBS and transferred to a Falcon 2056 tube. The cells were pelleted by centrifuging at 1200 rpm for 5 min followed by aspiration of the PBS wash. Cells were fixed in ice cold 70% ethanol with rapid but gentle mixing at a density of 1×10^6 cells/mL. After the cells were fixed in ethanol for 1 to 3 hr at 4°C, samples were stored at -20°C until analysis. For staining, the cells were centrifuged at 2500 rpm for 5 min to remove ethanol, washed one time with PBS and pelleted as above. The cells were resuspended in flow cytometric DNA staining reagent (0.1 mM EDTA [pH 7.4], 0.1% of Triton X-100, 0.05 mg/mL RNase A [50 units/mg], and 50μg/mL propidium iodide [PI] in PBS [pH 7.4]) before incubating overnight in the dark at 4°C. Fluorescence was assessed on a FACS Vantage (Beckton Dickinson) by excitation with an Argon laser at 488 nm and the emission was detected at 620 to 700 nm. Data were collected with Lysis II software and

the percentage of cells in each phase of the cell cycle was calculated with MPLUS software (Phoenix Flow). Apoptotic cells were determined as the percentage of cells with a DNA content of less than diploid.

PCR-based telomerase assay-Cells were harvested by trypsinization. After cell counting, the cells were centrifuged to remove trypsin solution. Cell pellets were washed with 10 mL PBS and then centrifuged to remove PBS. Cells were then suspended at 1×10^6 cells/mL in PBS and aliquoted into Eppendoff tubes. After cells were centrifuged and the PBS was carefully removed, the cell pellets were stored at -85°C. The telomerase assay was kindly performed by Dr. Wei Sun. The cell pellets were thawed and resuspended in 200 μL of 1× CHAPS lysis buffer/10^6 cells and left on ice for 30 min. The samples were centrifuged at 12,000 g for 20 min at 4°C. The cell lysates for each sample were aliquoted to several new tubes and stored at -85°C. The original lysates represent the concentration of 5000 cells/μL. Further dilution of cell lysates were adjusted based on the level of telomerase activity from individual cell lines. Telomerase activities were measured utilizing the TRAPeze™ Telomerase Detection Kit (Oncor, Gaithersburg, MD) which includes a primer of a 36 base pairs internal standard for amplification, thus 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 positive cell line control (MCF-7 breast carcinoma cells). The products of the TRAP assay were resolved by electrophoresis in a non-denaturing 12% polyacrylamide gel electrophoresis (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 Syber Green (Molecular Probes, Inc., Eugene, OR), and visualized using a 302 nm UV transilluminator. Images were captured and

analyzed by AlphaImager™ (Alpha Innotech Corporation, SanLeandro, CA).

Statistical analyses-Statistical analyses were conducted using the SPSS software. Data of cell proliferation were analyzed by two-way factorial analysis of variance (ANOVA). Differences in total nucleic acid content between control and treatment groups at specific culture periods were evaluated by multiple comparisons using Dunnett t-test. Differences were considered significant at $p<0.05$.

IV. RESULTS

A. Sphingosine and ceramide inhibit cell proliferation

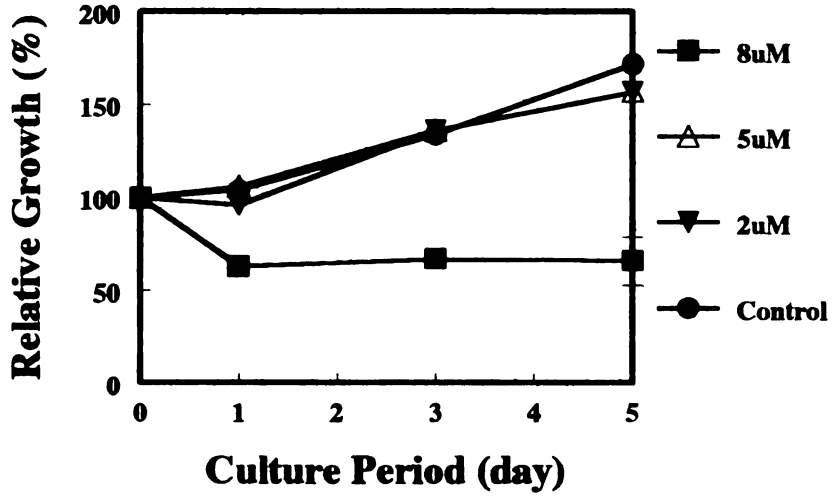
To assess the effects of sphingosine and ceramide on the proliferation of normal and tumorigenic breast cells, subconfluent cells were cultured with sphingosine and C_2-ceramide. The total nucleic acid content was quantitated as an index of cell number.

Sphingosine inhibits proliferation and causes death of Type I HBEC and tumorigenic breast cells, but not Type II HBEC at 8 μM-For Type I HBEC (Figure 5A), control cultures grew slowly over the culture period with total nucleic acid increasing about 70% in 5 days. Sphingosine at 2 and 5 μM did not effect cell proliferation; however, sphingosine at 8 μM reduced the nucleic acid concentration to about 60% of the corresponding control cultures at day 1 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.

For Type II HBEC (Figure 5B), the control 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.

The addition of sphingosine caused concentration- and time-dependent decreases in total nucleic acid concentration in all three tumorigenic breast cell lines (Figure 6). Sphingosine at 8 μM significantly inhibited the proliferation of and caused death of all of the tumorigenic breast cell lines. The transformed Type I HBEC line was the most sensitive, with 5 μM sphingosine reducing total nucleic acid content to about 50% compared with control at day 5 (Figure 6A). For MCF-7 cells and MDA-MB-231 cells, 5 μM sphingosine reduced total nucleic acid content to about 80% of controls at day 5 (Figure 6B and C).

A



B

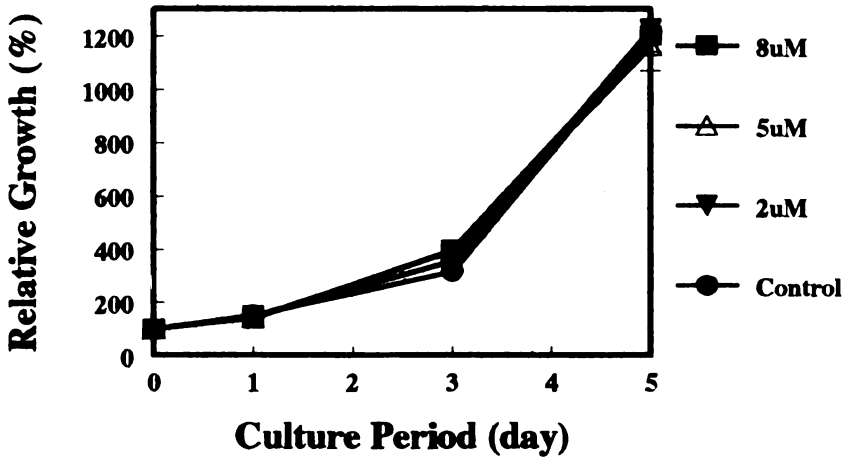


Figure 5. Sphingosine at 8 μM inhibits proliferation and causes death of Type I HBEC but not Type II HBEC. Type I (A) and II (B) HBEC (6×10^4) were cultured in 6-well dishes in triplicate and treated with sphingosine at day 0 and day 3. Total nucleic acid was measured by spectrophotometry (λ=260 nm) and used as an index of cell number. Results shown are mean ± SD (n=3). Standard deviations which are not visible are hidden by the symbols.

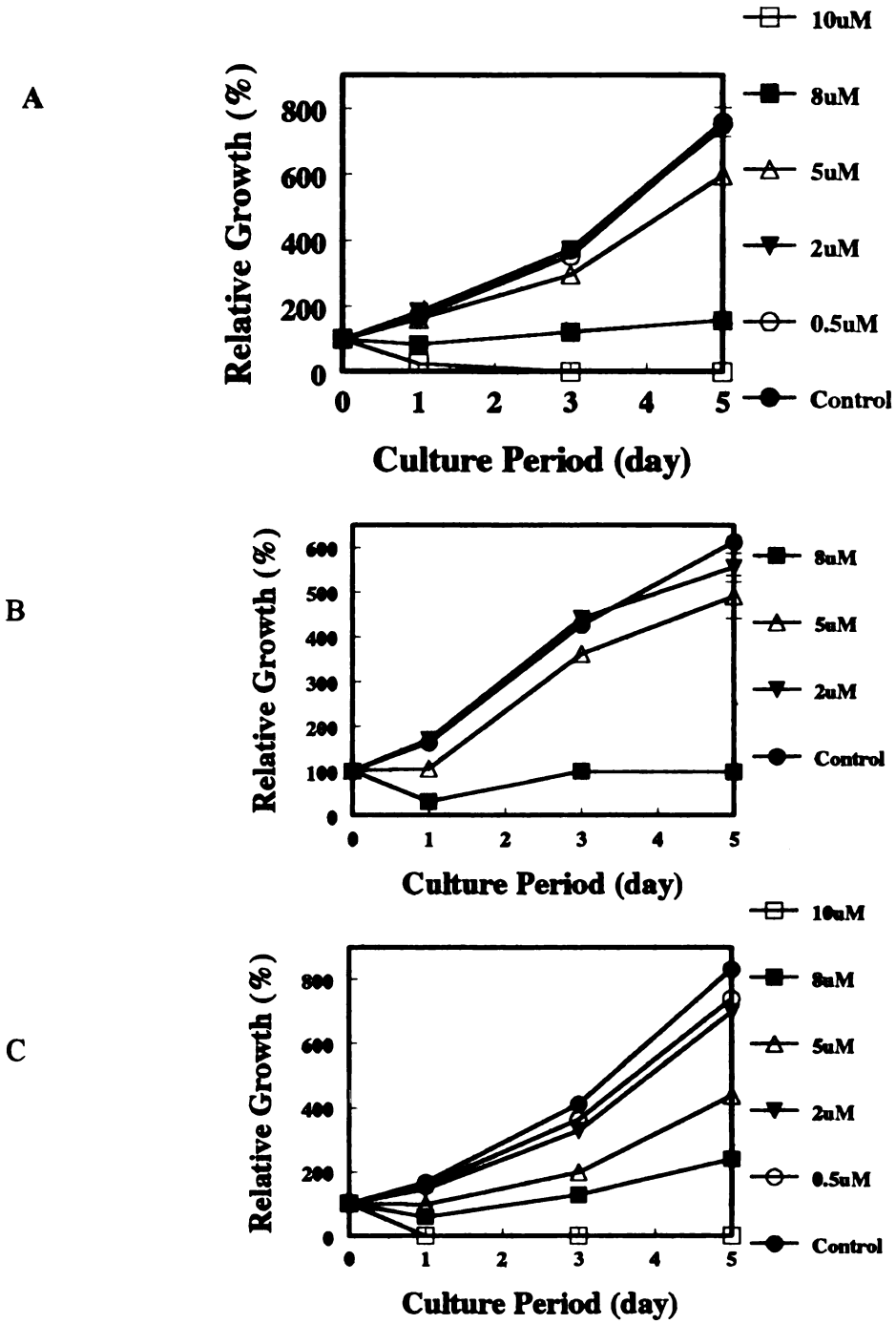


Figure 6. Sphingosine inhibits proliferation and causes death of tumorigenic breast cells. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells and transformed Type I HBEC (C)(6×10^4) were cultured in 6-well dish in triplicate and treated with sphingosine. Cell proliferation was assessed by total nucleic acid content. Results shown are mean ± SD (n=3). Standard deviations which are not visible are hidden by the symbols.

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Ceramide inhibits cell proliferation and causes death-For Type I HBEC, the total nucleic acid content for vehicle (ethanol) control culture nearly tripled in 5 days (Figure 7A). C_2-ceramide at 1 μM did not effect cell proliferation over 1 day; whereas, C_2-ceramide at 2 and 5 μM significantly inhibited cell proliferation and caused death within 1 day. C_2-ceramide at 5 μM reduced nucleic acid content to about 60% of the corresponding control at day 1 and to about 25% of the control by day 5. C_2-ceramide at 8 μM killed all of the cells by day 3 (data not shown). For Type II HBEC (Figure 7B), C_2-ceramide at 5 μM significantly inhibited cell proliferation and caused cell death within 1 day. For tumorigenic breast cells, C_2-ceramide was more potent than sphingosine as 5 μM C_2-ceramide completely inhibited cell proliferation in all three tested cell lines (Figure 8).

Sphingosine stereoisomers inhibit proliferation and cause death of transformed Type I HBEC-To study the structural requirements for sphingosine to cause cell death, three unnatural stereoisomers, D-*threo*, L-*threo* and L-*erythro*-sphingosine were examined together with D-*erythro*-sphingosine (Figure 9). All three unnatural stereoisomers of sphingosine were more potent than D-*erythro*-sphingosine in inhibiting proliferation and causing death of transformed Type I HBEC. L-*erythro*-sphingosine was the most potent.

Sphingosine and ceramide cause cell cycle arrest at G_0/G_1 or G_2/M-Flow cytometric analysis was used to study the effects of sphingosine and C_2-ceramide on cell cycle progression in Type I HBEC (Table 1 and Figure 10-11), MCF-7 cells (Table 2) and transformed Type I HBEC (Table 3). As shown in Table 1, control Type I HBEC exhibited normal homeostatic cell cycle distribution with 73.4 % of the cells in the G_0/G_1 phase and 11.3 % in the S (DNA synthesis) phase. Sphingosine at 10 μM caused cell cycle arrest at the G_0/G_1 phase within 1 day as indicated by an increase of cells in G_0/G_1 phase to 91.5% and a

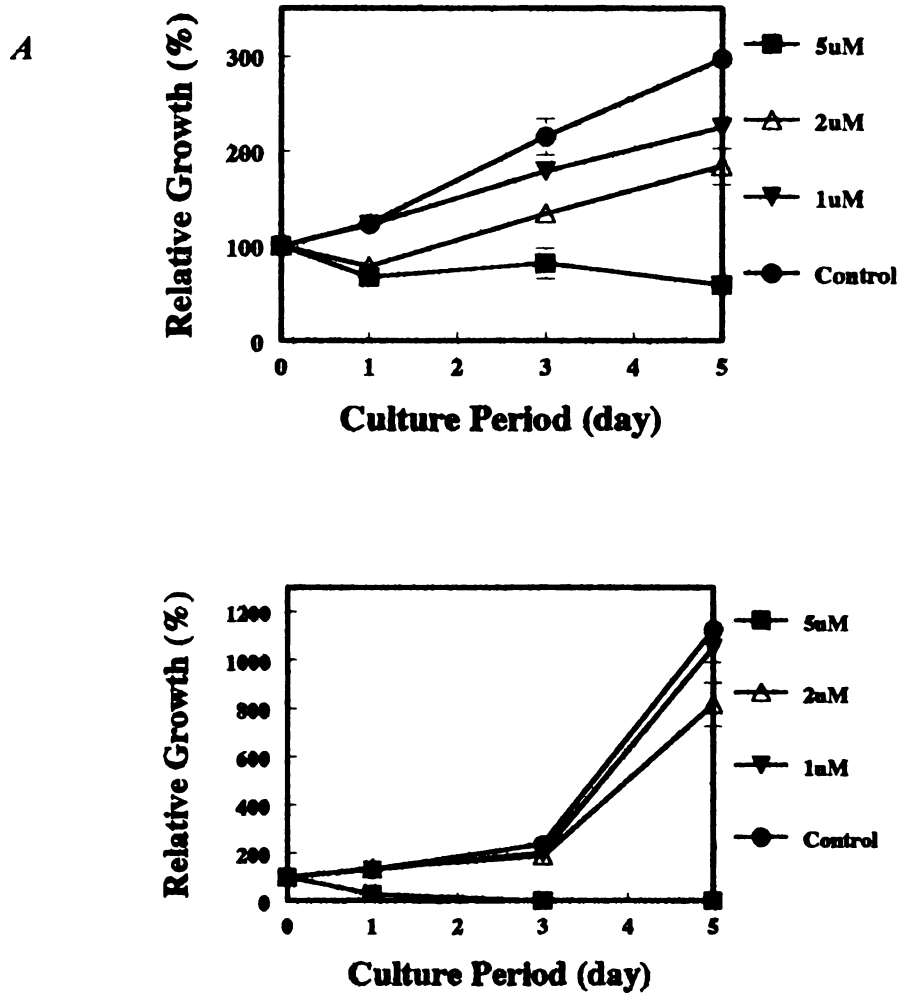
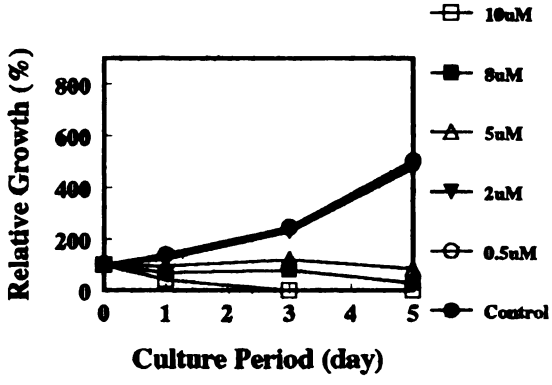


Figure 7. C_2-ceramide at 5 μM inhibits proliferation and causes death of Type I and II HBEC. Type I (A) and II (B) HBEC (6×10^4) were cultured in 6-well plates in triplicate and treated with C_2-ceramide. Cell proliferation was assessed by total nucleic acid content. Results shown are mean ± SD (n=3). Standard deviations which are not visible are hidden by the symbols.

A



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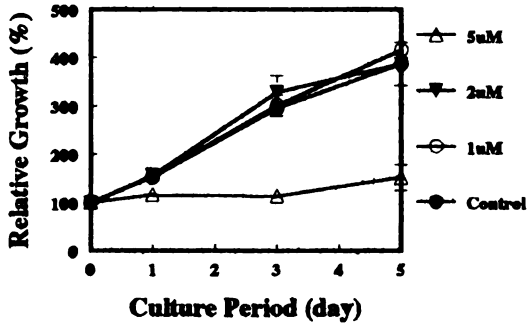

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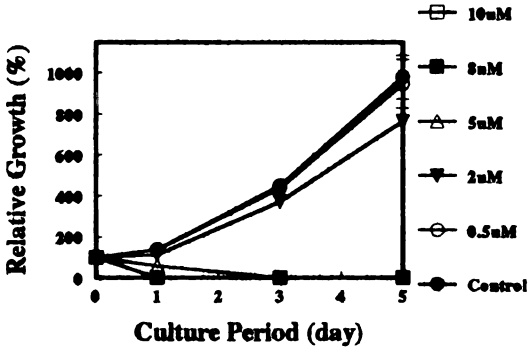

Figure 8. C_2-ceramide at 5 μM or higher concentrations inhibits proliferation and causes death of tumorigenic breast cells. MCF-7 (A) and MDA-MB-231 (B) breast cancer cells and transformed Type I HBEC (C)(6×10^4) were cultured in 6-well plates in triplicate and treated with C_2-ceramide. Cell proliferation was assessed by total nucleic acid content. Results shown are mean ± SD (n=3). Standard deviations which are not visible are hidden by the symbols.

Table 1. Sphingosine and ceramide affect cell-cycle distribution of Type I HBEC. Data are shown as percentage of the cells in each phase of cell cycle.

Treatments	**G_0 /G_1 phase (%)**	**S phase (%)**	**G_2/M phase (%)**
Control	73.4	11.3	15.4
Sphingosine (10 μM) 8 h	75.5	9.5	15.0
Sphingosine (10 μM) 24 h	91.5	2.0	6.5
C_2-ceramide (5 μM) 8 h	78.5	8.4	13.1
C_2-ceramide (5 μM) 24 h	77.7	10.2	12.0

Table 2. Sphingosine and ceramide affect cell-cycle distribution of MCF-7 breast cancer cell lines. Data are showed as percentage of the cells in each phase of cell cycle.

Treatments	**G_0/G_1 phase (%)**	**S phase (%)**	**G_2/M phase (%)**
Sphingosine (control)	43.9	29.8	26.3
Sphingosine (10 μM)	51.0	37.8	11.2
C_2-ceramide (control)	63.5	26.2	10.2
C_2-ceramide (10 μM)	79.1	10.0	10.9

Table 3. Sphingosine and ceramide affect cell-cycle distribution of transformed Type I HBEC. Data are showed as percentage of the cells in each phase of cell cycle.

Treatments	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
Sphingosine (control) 1d	35.8	24.5	39.7
Sphingosine (10 μM) 1d	39.5	25.3	35.2
C_2-ceramide (control) 1d	41.5	34.9	23.7
C_2-ceramide (10 μM) 1d	47.3	19.7	33.0
Sphingosine (control) 2d	47.3	29.1	23.6
Sphingosine (10 μM) 2d	46.6	22.8	30.5
C_2-ceramide (control) 2d	49.0	23.5	27.5
C_2-ceramide (10 μM) 2d	62.0	0.0	38.0

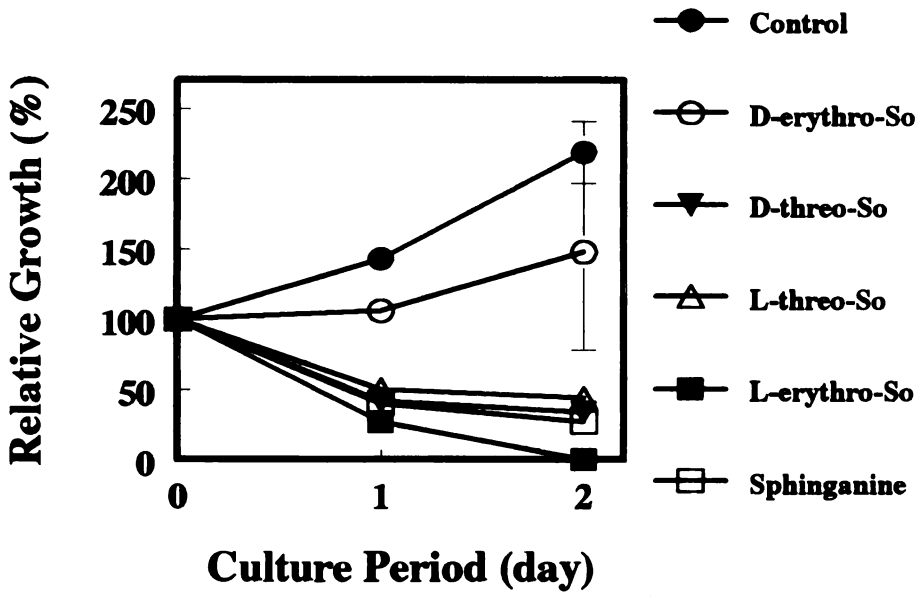

Figure 9. Sphingosine stereoisomers inhibit proliferation and cause death of tumorigenic breast cells. Transformed Type I HBEC (6×10^4) were cultured in 6-well plates and treated with 5 μM sphingosine stereoisomers. Cell proliferation was assessed by total nucleic acid content. Results shown are mean ± SD (n=3). Standard deviations which are not visible are hidden by the symbols.

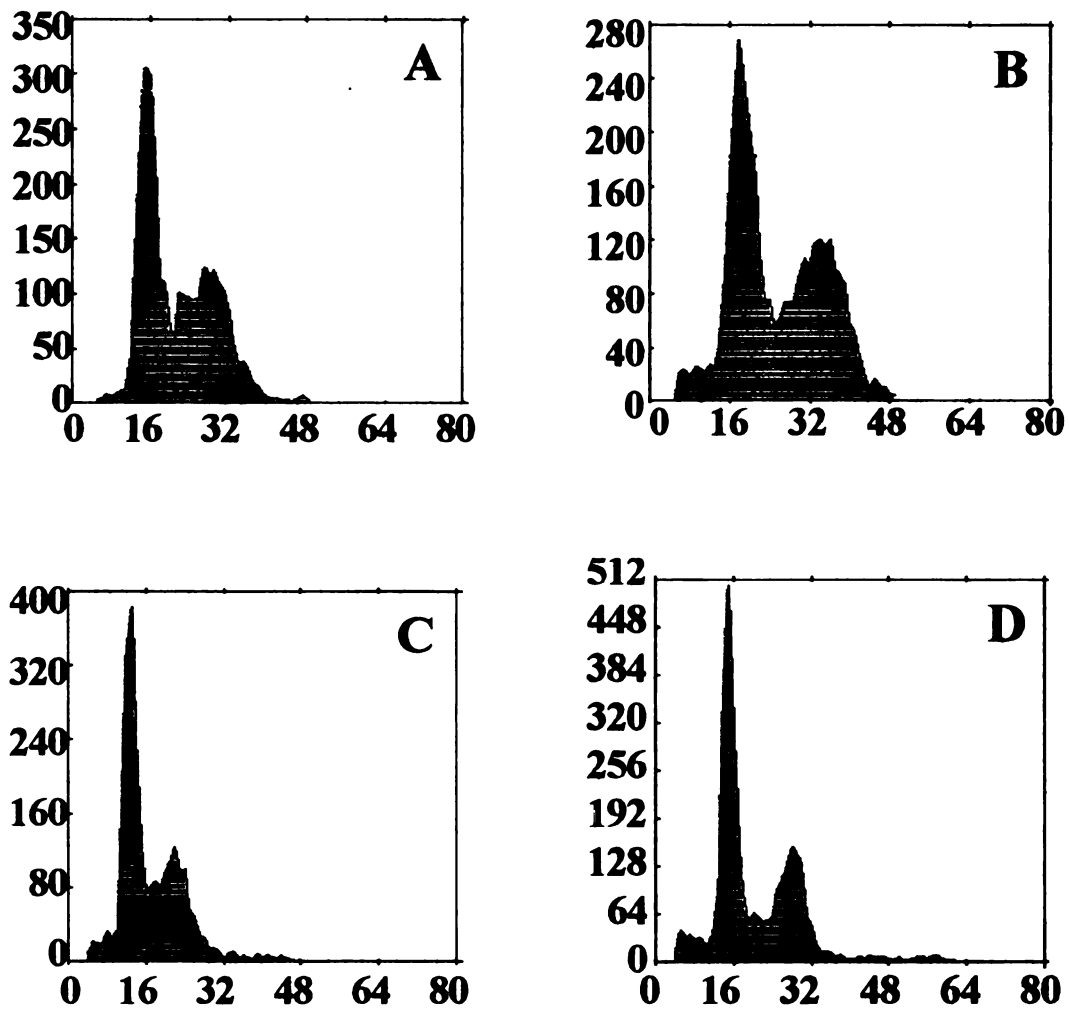

Figure 10. Flow cytometric analysis of transformed Type I HBEC cultured with sphingosine. Subconfluent transformed Type I HBEC were cultured with 0 (A and C) or 10 μM (B and D) sphingosine for 1 (A and B) or 2 (C and D) days. Cells were stained with propidium iodide to determine DNA content.

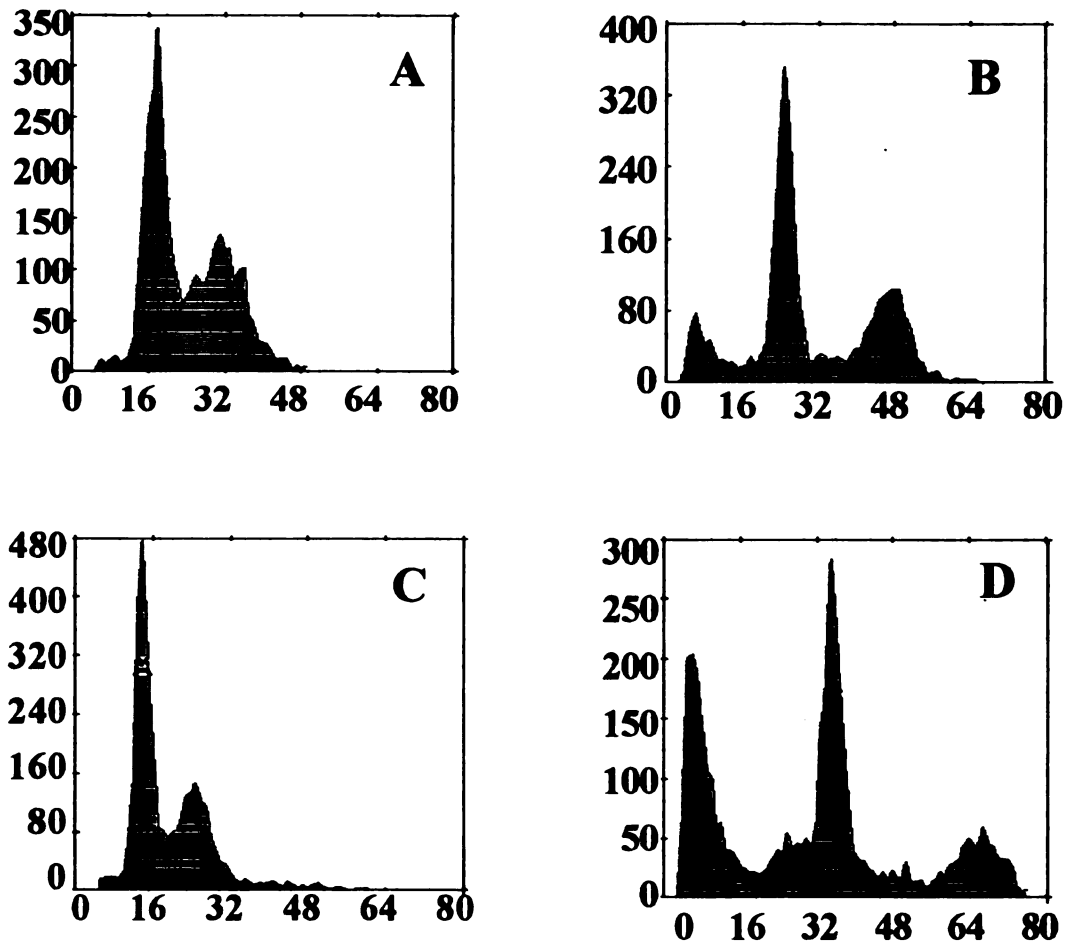

Figure 11. Flow cytometric analysis of transformed Type I HBEC cultured with C_2-ceramide. Subconfluent transformed Type I HBEC were cultured with 0 (A and C) or 10 μM (B and D) C_2-ceramide for 1 (A and B) or 2 (C and D) days. Cells were stained with propidium iodide for DNA content.

dramatic decrease of cells in S phase to 2.0%. C_2-ceramide at 5 μM, slightly increased cells in the G_0/G_1 phase to 77.7% and caused a slight decrease of cells in S phase to 10.2%.

For MCF-7 breast cancer cells, 10 μM sphingosine caused cells in the G_0/G_1 phase to increase from 43.9% to 51.0% and 10 μM C_2-ceramide caused cells in the G_0/G_1 phase to increase from 63.5% to 79.1% within 2 days. However, while C_2-ceramide decreased cells in S phase from 26.2% to 10.0%, sphingosine increased the cells in S phase from 29.8% to 37.8%.

For transformed Type I HBEC cultured with 10 μM C_2-ceramide for 2 days, cells in the G_0/G_1 phase increased from 49% to 62% with no cells in S phase. However, sphingosine increased cells in the G_2/M phase from 23.6% to 30.5% with cells in the S phase decreasing from 29.1% to 22.8%.

B. Sphingosine, but not C_2-ceramide, induces differentiation of Type I HBEC

Type I HEBC were cultured with and without various concentrations of sphingosine and C_2-ceramide. Then differentiation was measured by counting the numbers of Type II colonies and colonies of Type I surrounded by Type II HBEC. As shown in Table 4, control cells had a low rate of differentiation of 6.63% by day 5 and 7.76% by day 9. Cholera toxin (1 ng/mL), a positive control known to induce differentiation (Kao et al., 1995), significantly increased the number of Type II containing colonies to 62.26% by day 5 and 92.84% by day 9 without significant alteration in total colony number.

Sphingosine at concentrations from 1 to 3 μM significantly increased the frequency of colonies containing Type II HEBC starting at day 5 (Figure 12). This trend became more obvious at day 9. Also, the induction of differentiation of Type I to Type II HEBC was accompanied by concentration-dependent inhibition of colony forming efficiency, as shown

Table 4. Sphingosine causes differentiation of Type I HBEC. Type I HBEC (5×10^3) were cultured in 60 mm plates in triplicate and treated with various concentrations of sphingosine. Cholera toxin (CT) (1 ng/mL) was used as a positive control. Type I and Type II containing colonies were quantitated at day 5 and day 9 after first treatment.

Culture Peroid (days)	Treatments	Total Colonies	Type II HBEC containing colonies/ Total colonies (% of Type II HBEC containing colonies)
5	Control	392	26/392 (6.63%)
	Cholera toxin (1 ng/mL)	371	231/371 (62.26%)
	Sphingosine (1 μM)	356	49/356 (13.76 %)
	Sphingosine (2 μM)	187	39/187 (20.86 %)
	Sphingosine (3 μM)	32	7/32 (21.88%)
9	Control	322	25/322 (7.76%)
	Cholera toxin (1 ng/mL)	335	311/335 (92.84%)
	Sphingosine (1 μM)	350	92/350 (26.29%)
	Sphingosine (2 μM)	223	55/223 (24.66%)
	Sphingosine (3 μM)	43	10/43 (23.26%)

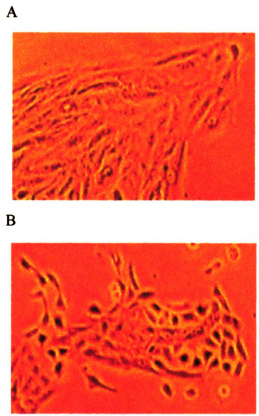

Figure 12. Sphingosine induces differentiation of Type I HBEC (photographs). Type I H BEC (1×10^4) were cultured in triplicate in 30 mm dishes with sphingosine at 0 (A) and 2 μM (B)sphingosine for 5 days.

Table 5. Ceramide does not cause differentiation of Type I HBEC. Type I HBEC (5×10^3) were cultured in 60 mm plates in triplicate and treated with various concentrations of C_2-ceramide. Cholera toxin (CT) (1 ng/mL) was used as a positive control. Type I and Type II containing colonies were quantitated at day 5 and day 9.

Culture Period (days)	Treatments	Total Colonies	Type II HBEC containing colonies/ Total colonies (% of Type II HBEC containing colonies)
8	Control	517	125/517 (24.18%)
	Cholera toxin (1 ng/mL)	462	250/462 (54.11%)
	C_2-ceramide (0.1 μM)	503	105/503 (20.87 %)
	C_2-ceramide (0.3 μM)	466	52/466 (11.16 %)
	C_2-ceramide (0.5 μM)	138	4/138 (2.90%)
12	Control	798	318/798 (39.85%)
	Cholera toxin (1 ng/mL)	857	670/857 (78.15%)
	C_2-ceramide (0.1 μM)	753	246/753 (32.67%)
	C_2-ceramide (0.3 μM)	705	160/705 (22.70%)
	C_2-ceramide (0.5 μM)	70	2/70 (1.96%)

by the decrease in the number of total colonies.

For C_2-ceramide (Table 5), control cells (0.1% ethenol) had a relatively high level of differentiation of 24.18% at day 8. Cells cultured with C_2-ceramide failed to show induction of differentiation compared with vehicle control. However, C_2-ceramide clearly caused concentration-dependent inhibition of colony forming efficiency, as indicated by a significant decrease in the number of total colonies.

C. Sphingosine and ceramide induce apoptosis.

Morphological changes characteristic of apoptosis, including cell shrinkage, membrane blebbing, chromatin condensation and the formation of apoptotic bodies were observed in Type I HBEC (Figure 13), transformed Type I cells (Figure 14) and MDA-MB-231 cells (Figure 15) treated with sphingosine and C_2-ceramide. To further investigate the mechanism of cell death, the presence of DNA fragmentation, a hallmark of apoptosis, was examined using agarose gel electrophoresis in cells cultured with or without C_2-ceramide. For Type I HBEC, control cultures had intact, high molecular weight DNA that remained at the top of the agarose gel (Figure 16-lane 2). DNA extracted from cells cultured with 10 μM C_2-ceramide for 1, 2 and 4 days was fragmented and showed a characteristic ladder pattern indicative of apoptosis (Figure 16-lanes 3, 4 and 5).

For transformed Type I HBEC cultured with or without sphingolipids as shown in Figure 17 and 18, both sphingosine and C_2-ceramide at 10 μM caused the formation of a DNA ladder within 1 day. C_2-ceramide at 5 μM, which killed cells in 3 days (Figure 8C), also induced cell death via an apoptotic pathway. A time-course study showed that the DNA ladder is formed after 7.5 hours, appeared in 1 day and persisted at 2 days (Figure 18).

For MDA-MB-231 breast cancer cells, sphingosine at 10 μM caused a DNA ladder

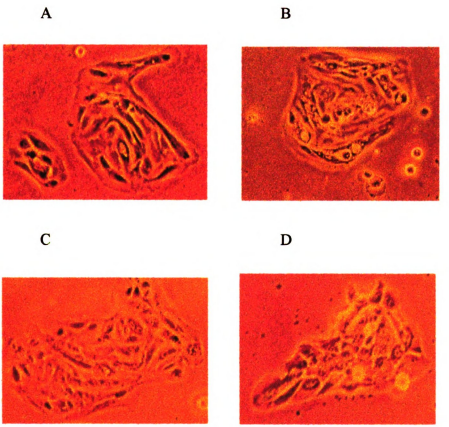

Figure 13. Sphingosine and ceramide cause morphological changes in Type I HBEC indicative of apoptosis (photographs). Type I HBEC were cultured with 0 (A) or 10 μM (B) sphingosine for 1 day and 0 (C) or 5 μM (D) C_2-ceramide for 3 days. Vehicle controls (A and C) have normal cell morphology. Membrance blebbing, chromatin condensation and apoptotic bodies are observed in treated cells (B and D).

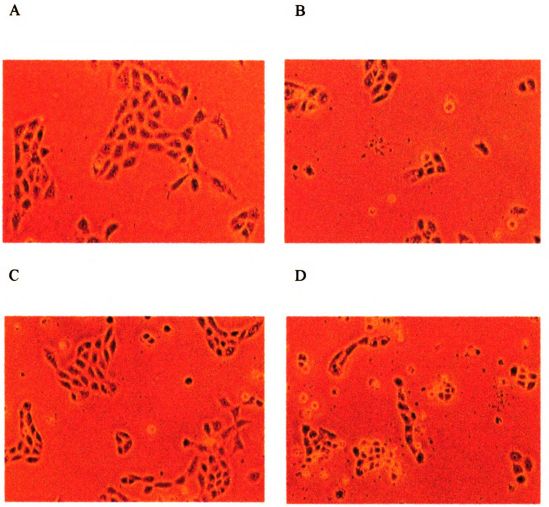

Figure 14. Sphingosine and ceramide cause morphological changes in transformed Type I HBEC indicative of apoptosis (photographs). Transformed Type I HBEC were cultured with 0 (A) or 8 μM (B) sphingosine and 0 (C) or 5 μM (D) C_2-ceramide for 1 day. Vehicle controls (A and C) have normal cell morphology. Membrance blebbing, chromatin condensation and apoptotic bodies are observed in treated cells (B and D).

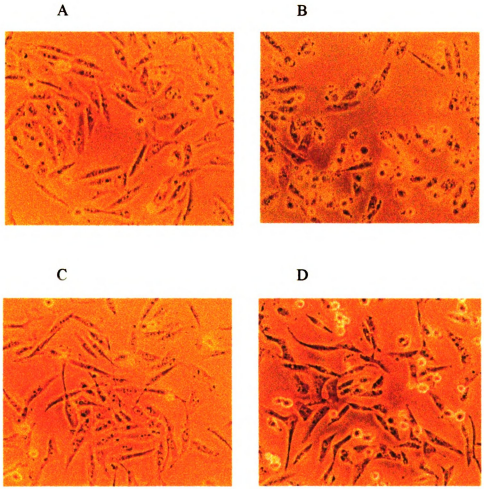

Figure 15. Sphingosine and ceramide cause morphological changes in MDA-MB-231 breast cancer cells indicative of apoptosis (photographs). MDA-MB-231 cells were cultured with 0 (A) or 10 μM (B) sphingosine for 12 hours and 0 (C) or 10 μM (D) C_2-ceramide for 1 day. Vehicle controls (A and C) have normal cell morphology. Membrance blebbing, chromatin condensation and apoptotic bodies are observed in treated cells (B and D).

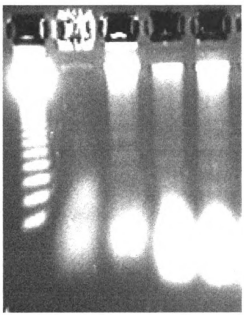

Figure 16. Ceramide cause internucleosomal DNA fragmentation in Type I HBEC. DNA of subconfluent Type I HBEC cultured with 10 μM C_2-ceramide for 1 (lane 3), 2 days (lane 4) or 4 days (lane 5) were extracted and run in 2 % agarose gel. Lane 1 shows DNA molecular marker and lane 2 is control at the 0 hour.

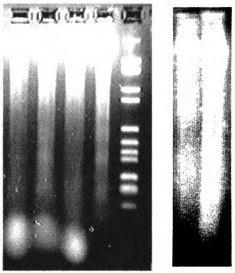

Figure 17. Sphingosine and ceramide cause internucleosomal DNA fragmentation in transformed Type I HBEC. DNA of subconfluent transformed Type I HBEC cultured without sphingolipid (lane 1, 3 and 6), with 10 μM sphingosine (lane 2) or 10 μM C_2-ceramide (lane 4) for 1 day or 5 μM C_2-ceramide (lane 7) for 3 days were extracted and run in 2 % agarose gel. Lane 5 shows DNA molecular marker.

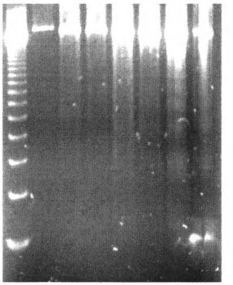

Figure 18. Sphingosine and ceramide cause time-dependent internucleosomal DNA fragmentation in transformed Type I HBEC. DNA of subconfluent transformed Type I HBEC cultured with 8 μM sphingosine (lane 3 and 5) or 10 μM C_2-ceramide (lane 4, 6 and 7) for 7.5 hours (lane 3 and 4), 1 day (lane 5 and 6) or 2 days (lane 7) were extracted and run in 2 % agarose gel. Lane 1 shows DNA molecular marker and lane 2 is control at the 0 hour.

starting at day 1 which became more intense after 2 days of treatment (Figure 19). C_2-ceramide at 10 μM caused a DNA ladder within 1 day which increased after 2 days of treatment, and, lower concentrations such as 5μM also caused a ladder pattern (Figure 20). However, MCF-7 cells treated with sphingosine and C_2-ceramide at cytotoxic doses failed to display a DNA ladder in agarose gel electrophoresis (data not shown).

Flow cytometric analysis confirmed the induction of apoptosis in transformed Type I cells treated with sphingosine or C_2-ceramide by a sharp hypodiploid pre-G_0/G_1 peak (Figure 10-11), which is formed by apoptotic cells with reduced DNA content. As shown in Table 6, cells in the pre-G_1 region (apoptotic region) increased from 1.06% to 4.75 upon treatment with sphingosine and from 2.44% to 15.22% for C_2-ceramide within 1 day and further increased upon longer treatment. These results provide strong evidence that sphingosine and C_2-ceramide induce apoptosis in Type I HBEC as well as in MDA-MB-231 and transformed Type I cells .

D. Sphingosine decreases telomerase activity in transformed Type I HBEC.

To explore the molecular mechanism of action of sphingosine, telomerase repeat amplification protocol (TRAP) was used to detect telomerase activity of transformed Type I HBEC cultured with sphingosine. In this assay, telomerase-synthesized extension products are amplified by polymerase chain reaction (PCR) and a ladder of products with 6 base increments starting at 50 nucleotides indicates positive telomerase activity (Kim et al., 1997). An internal control is included to enable quantitation of telomerase activity and identify false negative samples. As shown in Figure 19, after 2 days of incubation, control cells (lane 1) had telomerase activity and this was decreased by 5 μM sphingosine (lane 2). Telomerase activity was further reduced by incubation with sphingosine for 5 days (lane 3). The comparable

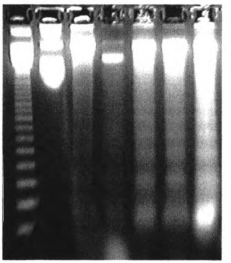

Figure 19. Sphingosine causes internucleosomal DNA fragmentation in MDA-MB-231 breast cancer cells. DNA of subconfluent MDA-MB-231 breast cancer cells cultured without sphingolipid (lane 2, 4 and 6) or with 10 μM sphingosine (lane 3, 5 and 7) for 12 (lane 2 and 3), 24 (lane 4 and 5) and 48 (lane 6 and 7) hours were extracted and run in 2 % agarose gel. Lane 1 shows DNA molecular marker.

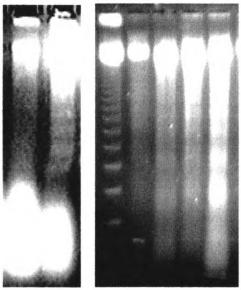

Figure 20. Ceramide causesinternucleosomal DNA fragmentation in MDA-MB-231 breast cancer cells. DNA of subconfluent MDA-MB-231 breast cancer cells cultured without sphingolipid (lane 1 and 4), with 10 μM C_2-ceramide for 1 day (lane 2) or 2 days (lane 6) or 5 μM C_2-ceramide for 2 days (lane 5) or 3 days (lane 7) were extracted and run in 2 % agarose gel. Lane 3 shows DNA molecular marker.

Table 6. Effects of sphingosine and ceramide on apoptosis of transformed Type I HBEC. Data are showed as percentage of apoptosis cells in total cell population.

Treatments	Apoptosis (%) 1d	Apoptosis (%) 2d
Sphingosine (control)	1.06	4.92
Sphingosine (10 μM)	4.75	7.11
C_2-ceramide (control)	2.44	2.48
C_2-ceramide (10 μM)	15.22	34.19

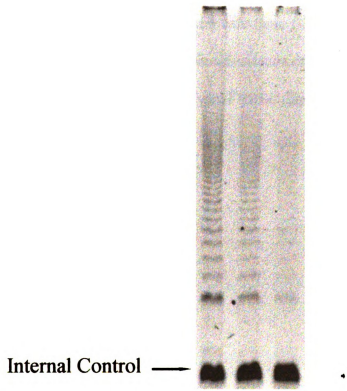

Figure 21. Sphingosine decreases telomerase activity in transformed Type I HBEC. Subconfluent cells were incubated with 0 (Lane 1) or 5 μM (Lane 2 and 3) sphingosine for 2 (Lane 1 and 2) or 5 (Lane 3) days. Cells were pelleted and analyzed by the TRAP assay. The PCR-amplified telomerase products were electrophoresed in 12 % polyacrylamide gel. Telomerase activity is visualized by the characteristic 6 base pair ladders.

internal standard indicates similar amplification efficiency.

E. Sphingosine causes dephosphorylation of retinoblastoma protein (Rb) in MCF-7 breast cancer cells but not Type II HBEC

Western blotting was used to examine the expression of retinoblastma protein (Rb) (Figure 20). For MCF-7 breast cancer cells (lane 1-3), sphingosine at 5 (lane 2) and 8 μM (lane 3) caused a dose-dependent dephosphorylation of Rb after 2 days. However, there was no change in Rb phosphorylation in Type II HBEC (lane 4-5) cultured with 5 μM sphingosine for 2 days.

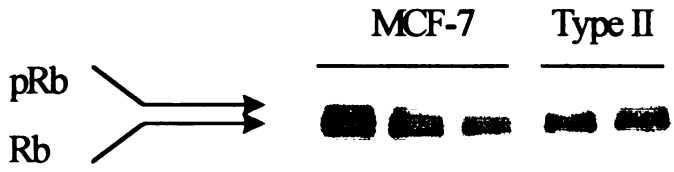

Figure 20. Sphingosine alters the expression of retinoblastoma protein (Rb) in MCF-7 breast cancer cells but not Type II HBEC. Subconfluent MCF-7 breast cancer cells (lane 1, 2 and 3) and Type II HBEC (lane 5 and 6) were cultured with 0 (lane 1 and 4) , 5 μM (lane 2 and 5) or 8 μM (lane 3) sphingosine for 3 days. Protein were extracted and analyzed by Western blot.

V. DISCUSSION

The dietary sphingolipid metabolites, sphingosine and ceramide, are signaling molecules that regulate cell proliferation, differentiation and apoptosis. Previous studies using breast cancer cells showed that these compounds inhibited proliferation and induced apoptosis (Gill *et al.*, 1997; Cai *et al.*, 1997; Zhang and Schroeder, 1998) which suggested that they may have chemotherapeutic potential. A unique aspect of the current study is that it is the first to evaluate the plausibility of using these sphingolipids as chemotherapeutic drugs by comparing their effects on the proliferation and death of tumorigenic breast cells to normal breast epithelial cells (Type II HBEC). Furthermore, since Type I HBEC have stem cell characteristics and are more susceptible to neoplastic transformation (*i.e.* the target cells for breast carcinogenesis) (Sun *et al.*, 1999), this study is also the first to evaluate the chemopreventive potential of sphingosine and ceramide by examining their effects on proliferation, differentiation and death of Type I HBEC.

A major finding of this study is that sphingosine preferentially inhibits proliferation and causes death of tumorigenic breast cell lines, while having no effect on the proliferation of Type II HBEC. Here, Type II HBEC, which have basal epithelial cell characteristics, were used as normal cell counterparts of breast cancer cells. Though sphingosine has previously been shown to induce apoptosis in SV40 transformed HUVEC and rat mesangial cells but not in their primary culture counterparts (Sweeney *et al.*, 1996), this study is the first to demonstrate differential effects of sphingosine on the proliferation of normal and tumorigenic breast cells. Sphingosine at 8 μM significantly inhibits proliferation and induces apoptosis of the tumorgenic breast cell lines in 5 days, while having no effect on the proliferation of Type II HEBC. The fact that tumorigenic breast cells are more sensitive to sphingosine than

Type II HBEC implies that this sphingoid base may be an ideal therapeutic agent with low potential for side effects. Moreover, since apoptosis induced by sphingosine is a well-regulated process, this sphingolipid is likely to also have the advantage of targeting individual cells without eliciting an inflammatory response in the surrounding normal tissue. The finding that the unnatural stereoisomers of sphingosine more potently inhibit proliferation and cause death of the transformed Type I HBEC than the natural D-*erythro* form is consistent with previous findings with MDA-MB-231 breast cancer cells (Zhang and Schroeder, 1999). This may be due to delayed metabolism of the unnatural sphingosine isomers. The fact that these structural analogs are more potent suggests that they may be more effective than D-*erythro*-sphingosine as chemotherapeutic agents.

Interestingly, compared to tumorigenic breast cells, Type I HBEC showed similar sensitivity to growth inhibition and death caused by sphingosine. This is not surprising since the phenotypes of many breast cancer cell lines (*e.g.* MCF-7 and MDA-MB-231) have been found to be similar to that of Type I rather than Type II HBEC (Kao *et al.*, 1995, 1997; Kang *et al.*, 1997). These include deficiency in gap junctional intercellular communication, expression of epithelial membrane antigen, cytokeratin 18, and estrogen receptors and growth promotion by fetal bovine serum. Since Type I HBEC have stem cell characteristics (Kao *et al.*, 1995; Sun *et al.*, 1999), these similarities are evidence for the stem cell theory or oncogeny as blocked or partially blocked ontogeny theory of carcinogenesis (Potter, 1978).

In contrast to sphingosine, C_2-ceramide inhibits proliferation of both normal Type II HBEC and tumorigenic breast cells with similar potency. Although C_2-ceramide seems to be more potent than sphingosine in that 5 μM C_2-ceramide causes similar cytotoxic effects as 8-10 μM sphingosine and C_2-ceramide is a strong inducer of apoptosis in tumorigenic

breast cells, C_2-ceramide does not appear to be an ideal chemotherapeutic agent unless future studies find it to have synergistic effects with other compounds. This might enable the use of C_2-ceramide at lower concentrations which are not toxic to normal tissue.

One possible mechanism by which sphingosine may affect cell proliferation is through the cell cycle regulating protein, retinoblastoma protein (Rb). Our results show that sphingosine induces a dose-dependent dephosphorylation of Rb in MCF-7 breast cancer cells at concentrations which are growth inhibitory. This is consistent with previous findings in lymphoblastic leukemia MOLT-4 cells (Chao *et al*, 1992). Furthermore, our studies demonstrate that concentrations of sphingosine which inhibit growth and cause Rb deposphorylation in MCF-7 breast cancer cells, do not affect proliferation or Rb phosphorylation in normal Type II HBEC.

Sphingosine decreased telomerase activity in tumorigenic Type I HBEC which is consistent with its effect on nasopharygeal cancer cells (Ku *et. al*, 1997). The close correlation between differentiation and suppression of telomerase activity suggests that sphingosine may induce the differentiation of tumorigenic Type I HBEC. Thus, sphingosine might provide additional therapeutic value by reducing the malignance of breast cancer. There are several mechanisms by which sphingosine could decrease telomerase activity. *C-myc* has been shown to activate the transcription of telomerase reverse transcriptase (hTERT) (Wu *et al.*, 1999). Therefore, sphingosine may act via its ability to inhibit *c-myc* expression (Ohta *et al*, 1995). In addition, protein kinase C inhibitors have been shown to inhibit telomerase activity (Ku *et al.*, 1997). Thus, an alternative mechanism by which sphingosine could suppress telemorase activity is via its ability to inhibit protein kinase C (Hannun *et al.*, 1986).

Another major finding of this study is that sub-lethal concentrations of sphingosine induce differentiation of Type I to Type II HBEC. This was observed as early as 5 days and at non-cytotoxic concentrations of 1-3 μM. For C_2-ceramide, however, no induction of differentiation was observed. This may have been due to the relatively high background of differentiation for the vehicle control. Like sphingosine, higher concentration of C_2-ceramide caused cell death. This suggests that C_2-ceramide is a strong inducer of death rather than differentiation for breast epithelial cells.

Both sphingosine and C_2-ceramide inhibited proliferation and caused death of Type I HBEC and C_2-ceramide caused cell death by inducing apoptosis. Sphingosine more potently inhibited proliferation of Type I HBEC than Type II HBEC; whereas, C_2-ceramide inhibited proliferation of both Type I and Type II with similar potency. Since Type I HBEC have been shown to be more susceptible to neoplastic transformation (*i.e.* target cells of carcinogenesis) (Kao *et al.*, 1995; Sun *et al.*, 1999), the ability of sphingosine to inhibit cell proliferation and to induce differentiaion at non-cytotoxic concentrations clearly suggests the lipid is a potential chemopreventive agent for breast cancer (Hsieh and Chang, 1999)..

The mechanism by which sphingosine induces differentiation is not clear. One possible pathway is by causing an increase in cellular cAMP similar to that brought about cholera toxin (Kao *et al.*, 1995). Alternatively, sphingosine might stimulate differentiation by influencing protein kinase C and/or *c-myc* expression. Vitamin D_3 has been shown to induce differentiation by affecting the expression of protein kinase C which phosphorylates a c-*myc* intron element and down-regulates c-*myc* expression (Simpson *et al.*, 1998).

VI. SUMMARY

This study evaluated the potential of sphingosine and C_2-ceramide as chemotherapeutic and chemopreventive agents for breast cancer by examining their effects on proliferation, differentiation and apoptosis of both normal and tumorigenic breast cells. Sphingosine was found to: 1) preferentially inhibit the proliferation and to cause death of Type I HBEC with stem cell characteristics as well as tumorigenic breast cell lines as compared with Type II HBEC with basal cell characteristics; 2) induce apoptosis in tumorigenic breast cells; and 3) induce differentiation of Type I HBEC to Type II HBEC at non-cytotoxic doses. Inhibition of cancer cell proliferation by sphingosine was accompanied by dephosphorylation of Rb and inhibition of telomerase activity. These data suggest that sphingosine has the potential to be used as a chemotherapeutic and chemopreventive agent for human breast cancer. C_2-ceramide had comparable inhibitory effects on proliferation of both normal Type II HBEC and tumorigenic breast cells and was capable of inducing apoptosis in both Type I HBEC and tumorigenic breast cells but did not induce differentiation of Type I HBEC to Type II HBEC. Thus, C_2-ceramide does not appear to be an ideal chemopreventive or chemotherapeutic agent for human breast cancer.

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