Sphingoid Bases and Ceramide Induce Apoptosis in HT-29 and HCT-116 Human Colon Cancer Cells

EUN HYUN AHN AND JOSEPH J. SCHROEDER[1]

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48824-1224

Complex dietary sphingolipids such as sphingomyelin and glycosphingolipids have been reported to inhibit development of colon cancer. This protective role may be the result of turnover to bioactive metabolites including sphingoid bases (sphingosine and sphinganine) and ceramide, which inhibit proliferation and stimulate apoptosis. The purpose of the present study was to investigate the effects of sphingoid bases and ceramides on the growth, death, and cell cycle of HT-29 and HCT-116 human colon cancer cells. The importance of the 4,5-*trans* double bond present in both sphingosine and C_2-ceramide (a short chain analog of ceramide) was evaluated by comparing the effects of these lipids with those of sphinganine and C_2-dihydroceramide (a short chain analog of dihydroceramide), which lack this structural feature. Sphingosine, sphinganine, and C_2-ceramide inhibited growth and caused death of colon cancer cells in time- and concentration-dependent manners, whereas C_2-dihydroceramide had no effect. These findings suggest that the 4,5-*trans* double bond is necessary for the inhibitory effects of C_2-ceramide, but not for sphingoid bases. Evaluation of cellular morphology via fluorescence microscopy and quantitation of fragmented low-molecular weight DNA using the diphenylamine assay demonstrated that sphingoid bases and C_2-ceramide cause chromatin and nuclear condensation as well as fragmentation of DNA, suggesting these lipids kill colon cancer cells by inducing apoptosis. Flow cytometric analyses confirmed that sphingoid bases and C_2-ceramide increased the number of cells in the A_0 peak indicative of apoptosis and demonstrated that sphingoid bases arrest the cell cycle at G_2/M phase and cause accumulation in the S phase. These findings establish that sphingoid bases and ceramide induce apoptosis in colon cancer cells and implicate them as potential mediators of the protective role of more complex dietary sphingolipids in colon carcinogenesis. [Exp Biol Med Vol. 227(5):345–353, 2002]

Key words: sphingosine; sphinganine; ceramide; colon cancer cells; apoptosis

R ecently, dietary sphingolipids have gained attention for their potential to protect against the development of colon cancer. Sphingolipids are a family of compounds that have a long-chain (sphingoid) base backbone and include free sphingoid bases (sphingosine and sphinganine), ceramides, sphingomyelins, cerebrosides, sulfatides, and gangliosides (1). Several studies have been conducted with milk sphingomyelin, synthetic sphingomyelin, synthetic dihydrosphingomyelin, as well as milk glycosphingolipids to determine if the sphingolipids protect CF1 mice from 1,2-dimethylhydrazine (DMH)-induced colon cancer (2–5). The results showed that sphingomyelin at 0.1% of the diet caused a higher percentage of adenomas and lower percentage of the more advanced adenocarcinomas (3). Interestingly, synthetic dihydrosphingomyelin (*N*-palmitoyl-dihydrosphingomyelin) more potently reduced the number of aberrant colonic foci than synthetic sphingomyelin (*N*-palmitoylsphingomyelin) and milk sphingomyelin, suggesting that the 4,5-*trans* double bond, which is absent in dihydrosphingomyelin, is not required for the suppression of colon carcinogenesis (4). Furthermore, major milk glycosphingolipids, including glucosylceramide, lactosylceramide, and ganglioside GD_3, effectively reduced aberrant colonic foci in CF1 mice treated with DMH (5). In addition, diets supplemented with ceramide, sphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD_3 reduced the number of tumors in all regions of the intestine in multiple intestinal neoplasia (Min) mice with a truncated adenomatous polyposis coli (APC) gene product (6). Therefore, the inhibitory effects of sphingolipids are not altered by the complexity of the sphingolipid head groups and are not limited to the chemically induced colon cancer animal model.

Although evidence is now emerging which suggests

that dietary sphingolipids may protect against the development of colon cancer, little is known about the mechanism. Sphingolipid metabolites such as ceramide and sphingoid bases play important roles in regulating cellular behavior and they have been implicated as putative second messengers in signaling pathways (1). For example, ceramide induces apoptosis in JB-6 tumor cells (7) and HT-29 human colon cancer cells (8), whereas sphingosine and its methylated derivative N,N-dimethyl sphingosine induce apoptosis in a variety of human cancer cells, including CMK-7, HL-60, U-937, HRT-18, MKN-74, and COLO-205 cells (9). Therefore, a possible mechanism by which complex dietary sphingolipids may reduce colon carcinogenesis is via digestion to bioactive metabolites such as ceramide and sphingoid bases.

The purpose of the present study was to systematically examine the effects of sphingoid bases and ceramides on the growth, apoptotic cell death, and cell cycle of HT-29 and HCT-116 human colon cancer cells. Both cell lines show epithelial morphology, are tumorigenic *in vivo*, and are commonly used to study chemotherapy (10), but have different genetic mutations. HT-29 cells carry mutations in APC (11) and *p53* (12, 13), but have normal β-*catenin* (11) and *ras* proto-oncogene (14) and express catalytically inactive cyclooxygenase-2 (COX2) (15). In contrast, HCT-116 cells carry mutations in β-*catenin* (16) and *ras* (17), but have normal APC (18, 19) and *p53* (20) and do not express COX2 (21). In order to evaluate the significance of the 4,5-*trans* double bond, the effects of sphingosine and the cell-permeable ceramide analog C_2-ceramide were compared with those of sphinganine and C_2-dihydroceramide, which lack this structural feature (Fig. 1). Morphological and biochemical features of apoptosis were assessed via fluorescence microscopy, quantitation of fragmented low-molecular weight DNA using the diphenylamine assay, and flow cytometry.

Materials and Methods

Chemicals and Reagents. Sphingolipids were obtained from Matreya (Pleasant Gap, PA). All other chemicals were from Sigma (St. Louis, MO) unless otherwise indicated.

Sphingolipid Treatment. Sphingosine and sphinganine were prepared as 1:1 complexes with bovine serum albumin (BSA) dissolved in phosphate-buffered saline (PBS), whereas C_2-ceramide and C_2-dihydroceramide (cell-permeable short-chain analogs of naturally occurring ceramide and dihydroceramide; Fig. 1) were dissolved in ethanol.

Cell Culture. HT-29 and HCT-116 human colon cancer cell lines were purchased from American Type Culture Collection (Manassas, VA). Stock cultures of HT-29 and HCT-116 cells were cultured in 100-mm dishes (Corning, Cambridge, MA) containing Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 3.5 g of
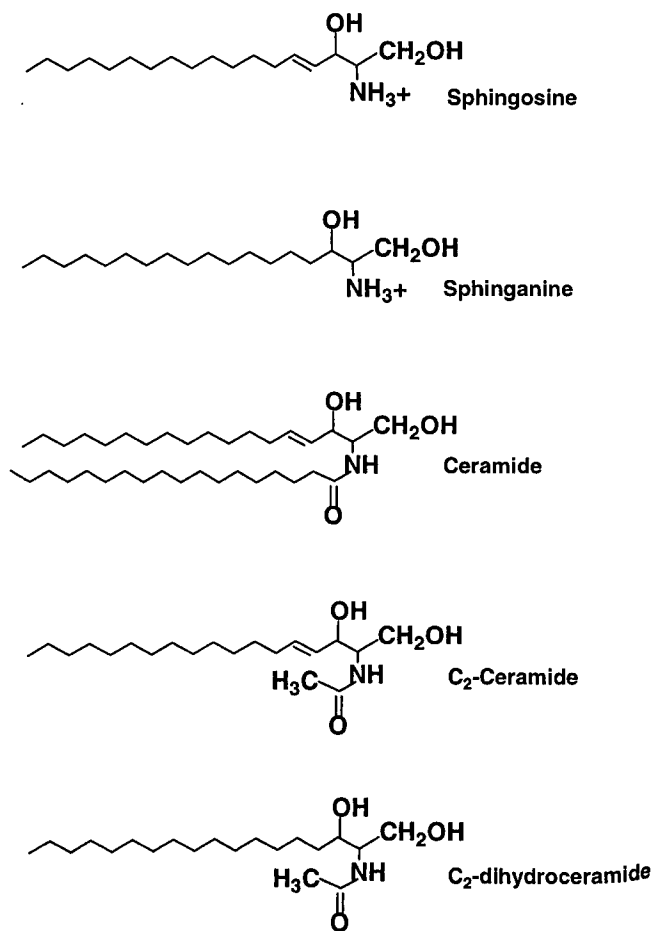

Figure 1. Structures of sphingosine, sphinganine, ceramide, C_2-ceramide, and C_2-dihydroceramide.

glucose/liter and 2.5 ml of penicillin-streptomycin/liter (Invitrogen) at 37°C and 5% CO_2. All experiments were performed using cells with from passage 20 or less.

Assessment of Cell Proliferation. To assess the effects of sphingolipids on cell growth and death, total nucleic acids were measured as previously described (22) and were used as an index of cell number. Briefly, HT-29 or HCT-116 cells were seeded at a density of 3×10^5 cells/ml in 6-well dishes and were cultured with 2 ml of DMEM with 10% FBS for 24 or 36 hr to ensure that cells were in log phase before treatment with sphingolipids. Then, the medium was replaced with DMEM supplemented with 1% FBS. Various concentrations of sphingolipids were added directly to each dish, and the cells were cultured for up to 48 hr. Following treatment, the medium and dead floating cells were removed, the viable attached cells were rinsed with 1 ml of PBS, and then the cells were lysed with 1 ml of 0.1 M NaOH. Total nucleic acid concentration was determined by measuring absorbance of the cell lysate at 260 nm using a Gene Quant-RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ).

Fluorescence Microscopic Detection of Apoptotic Cells. To determine whether sphingolipids kill cells via apoptosis, cells were seeded at a density of 2×10^5 cells/ml in 6-well dishes and were cultured with sphingo-

lipids at 20 μM for 24 hr. Both attached and detached cells were collected, stained with acridine orange and ethidium bromide, and examined via fluorescence microscopy (23). Acridine orange intercalates into DNA and causes the DNA to appear green. It also binds to RNA but cannot intercalate so that the RNA stains red-orange. Ethidium bromide is only taken up by nonviable cells and intercalates into DNA, causing it to appear orange, but binds only weakly to RNA. Thus, dead cells will have bright orange chromatin because the ethidium bromide overwhelms the acridine orange. Cells were photographed using a fluorescence microscope equipped with a camera under $\times 10$ plus $\times 40$ magnification with 400/490 nm excitation and 520 nm emission (Nikon Labophoto; Nikon Instrument Group, Garden City, NY). Apoptotic and non-apoptotic cells were classified by the differences in their chromatin organization as previously described (24).

Isolation of Fragmented Low-Molecular Weight DNA.
Cells were seeded at 2×10^5 cells/ml and were cultured with sphingolipids at 50 μM for 48 hr. After treatments, both attached and detached cells were collected and pelleted via centrifugation (150g for 5 min), and the supernatant was removed. Lysis buffer at 200 μl was added, and the cells were incubated for 10 min at 4°C. After centrifugation (14,000g for 30 min at 4°C), the supernatant containing fragmented DNA was transferred into a new 1.5-ml Eppendorf tube. Pellets were resuspended in 150 μl of Tris-EDTA (TE) buffer. Cells were incubated with 2 μl of 40 mg/ml RNase A at 37°C for 1 hr and 4 μl of 40 mg/ml Proteinase K (Invitrogen) at 37°C for 1 hr.

Quantitation of Fragmented Low-Molecular Weight DNA Using the Diphenylamine Assay.
Fragmented DNA was quantitated via the diphenylamine assay (25). Briefly, 108 μl of 5 M perchloric acid was added to samples (final concentration of perchloric acid was 1 M) and samples were heated at 70°C for 15 min. Diphenylamine reagent (1.5 g of diphenylamine, 1.5 ml of concentrated sulfuric acid, and 0.5 ml of 1.6% aqueous acetaldehyde in 100 ml of glacial acetic acid) at 1076 μl was then added and the samples were incubated at 30°C for 15 to 18 hr for color development. Salmon sperm DNA (Invitrogen) was used as a standard. Absorbance was measured at 600 nm and the percentage of fragmented DNA was calculated as (fragmented DNA/total DNA) $\times$ 100.

Flow Cytometric Analysis of Cell Cycle and Population.
Flow cytometric analysis was performed as described by Telford et al. (26) with some modifications. Cells were seeded at a density of 2×10^5 cells/ml and were cultured with sphingolipids at 35 μM for 24 hr. After treatments, both attached and detached cells were collected, trypsinized, and filtered through a 40-μm nylon sieve. Following centrifugation (150g for 5 min), the supernatant was removed and PBS (pH 7.4) containing 50% FBS was added. Cells at 10^6 to 2×10^6 cells/tube were prepared and fixed with 70% cold ethanol at a final concentration of 50% to 53%. After cells were stored at 4°C for 1 to 5 hr, cells were

pelleted by centrifugation (150g for 5 min) and resuspended in 500 μl of PBS containing 4% FBS (27). One milliliter of DNA staining solution (4% FBS, 0.1% Triton X-100, 100 μM EDTA, 0.05 mg/ml RNase A [50 units/mg], and 50 μg/ml propidium iodide in PBS [pH 8.0]) was then added and the cells were kept at 4°C for 1 to 2 hr before reading using a FACS Vantage Flow Cytometer (Becton Dickinson, San Jose, CA). The total number of cells analyzed for each sample was 5000.

The A_0 peak represents apoptotic cells and is the hypodiploid area (sub-G_0/G_1) to the left of the G_0/G_1 diploid peak. The percentage of cells in the A_0 peak was estimated with the FCS Express version 1.0 software (De Novo Software, Thornhill, Ontario, Canada). The percentages of cells in G_0/G_1, S, and G_2/M phases were determined using 'Win cycle', a multicycle DNA content and cell cycle analysis software (Phoenix Flow Systems, San Diego, CA). The Win cycle software program does not include any cell populations outside of standard cell cycle populations when calculating areas, thus excluding sub-G_0/G_1 data.

Statistical Analyses.
Data for total nucleic acids assay were analyzed by two-way factorial analysis of variance (ANOVA). After application of ANOVA, the significance of differences in the means between control and treatment groups at specific culture periods were evaluated by multiple comparisons using the Bonferroni method. Data for quantitation of fragmented DNA using the diphenylamine assay were analyzed by one-way ANOVA. Differences were considered significant at $P < 0.05$.

Results

Sphingosine, Sphinganine, and C_2-Ceramide Inhibit Growth and Cause the Death of HT-29 and HCT-116 Human Colon Cancer Cells.
To determine the effects of sphingolipids on the growth and death of human colon cancer cells, subconfluent HT-29 and HCT-116 cells were cultured with sphingosine, sphinganine, C_2-ceramide, or C_2-dihydroceramide and then total nucleic acids were determined as an index of cell number (22). For HT-29 cells, total nucleic acids in control cultures doubled over 36 to 48 hr (Fig. 2). Sphingosine, sphinganine, and C_2-ceramide each caused concentration- and time-dependent decreases in proliferation of HT-29 cells. Specifically, the addition of sphingosine (Fig. 2) at 20 and 50 μM significantly reduced total nucleic acid concentrations within 24 hr by 47% and 62%, respectively ($P < 0.05$) compared with the corresponding control. Sphinganine (Fig. 2) at 50 μM significantly ($P < 0.05$) reduced total nucleic acid concentrations within 24 hr by 20%, 28%, and 80%, respectively, compared with the corresponding control. C_2-ceramide (Fig. 2) also caused a significant ($P < 0.05$) reduction in the total nucleic acid concentrations at 20 and 50 μM within 48 hr. Culture with C_2-ceramide at 50 μM for 48 hr completely killed all cells. In contrast to the sphingoid bases and C_2-ceramide, C_2-dihydroceramide had no effect
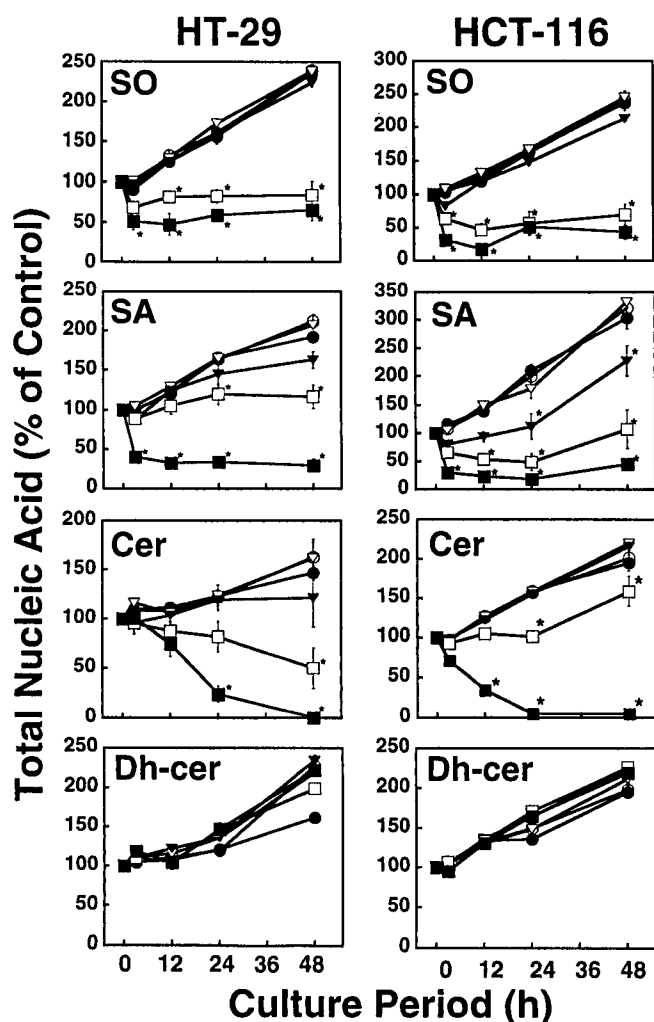
HT-29 **HCT-116**

Culture Period (h)

Figure 2. Effects of sphingoid bases and ceramides on the growth and death of HT-29 and HCT-116 human colon cancer cells. Subconfluent cells were cultured with sphingosine (SO), sphinganine (SA), C_2-ceramide (Cer), and C_2-dihydroceramide (Dh-cer) at 0 (●), 1 (○), 5 (▽), 10 (▼), 20 (□), and 50 (■) μM for 3, 12, 24, or 48 hr. Total nucleic acids were measured as an index of cell number. Results are expressed as a percentage of the control value at 0 hr. Data are from two experiments and represent mean ± SEM ($n = 8$). Where an error bar is not shown, 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.

on proliferation of HT-29 cells at concentrations as high as 50 μM (Fig. 2).

Total nucleic acids in control cultures of HCT-116 cells doubled within 24 to 36 hr, indicating slightly more rapid growth than for HT-29 cells (Fig. 2). Similar to their effects on HT-29 cells, sphingosine, sphinganine, and C_2-ceramide caused concentration- and time-dependent decreases in proliferation of HCT-116 cells. Specifically, addition of sphingosine (Fig. 2) at 20 and 50 μM significantly ($P < 0.05$) reduced total nucleic acid concentrations within 24 hr by 65% and 68%, respectively, compared with the corresponding control. Sphinganine (Fig. 2) at 10, 20, or 50 μM significantly ($P < 0.05$) reduced total nucleic acid concentrations within 24 hr by 46%, 77%, and 91%, respectively, compared with the corresponding control. C_2-ceramide at

20 μM also caused a significant ($P < 0.05$) reduction in total nucleic acid concentrations within 24 hr by 36%, and C_2-ceramide at 50 μM killed all of the HCT-116 cells within 24 hr (Fig. 2). Unlike the sphingoid bases and C_2-ceramide, C_2-dihydroceramide did not inhibit proliferation at concentrations as high as 50 μM (Fig. 2).

Fluorescence Microscopic Detection of Apoptotic Cells in HT-29 and HCT-116 Cells. The proliferation studies demonstrated that the higher concentrations of sphingoid bases and C_2-ceramide not only inhibited proliferation, but also caused the death of the colon cancer cells. To determine whether the sphingolipids kill the cells by inducing apoptosis, chromatin organization of HT-29 and HCT-116 cells was evaluated by staining with acridine orange and ethidium bromide using fluorescence microscopy (23, 24). Sphingosine, sphinganine, and C_2-ceramide at 20 μM for 24 hr each caused characteristic apoptotic morphological changes in both HT-29 and HCT-116 cells (Fig. 3). These included viable cells with apoptotic nuclei, which contain bright green chromatin with a highly condensed or fragmented structure as well as nonviable cells with apoptotic nuclei, which contain orange chromatin with a highly condensed or fragmented structure. The condensed and fragmented material is uniformly stained by the acridine orange and shows small bright circles with the overall cytoplasm not as bright as the cytoplasm of non-apoptotic control cells. In comparison, C_2-dihydroceramide had no effect and viable cells with normal nuclei contained bright green chromatin similar to control cells (Fig. 3).

Quantitation of Fragmented Low-Molecular Weight DNA Using the Diphenylamine Assay. To assess the percentage of cells that undergo apoptosis in response to sphingolipids, both attached and detached cells were collected 48 hr after treatment with each sphingolipid at 50 μM, and internucleosomal fragmented low-molecular weight DNA was quantitated using the diphenylamine assay (25). For HT-29 cells, control cultures had ~13% fragmented DNA (Fig. 4). Sphingosine, sphinganine, and C_2-ceramide caused significantly greater DNA fragmentation at 21%, 31%, and 27%, respectively ($P < 0.05$). In contrast, DNA fragmentation in cells cultured with C_2-dihydroceramide was 17%, which was comparable with controls (Fig. 4).

Results were similar for HCT-116 cells, although the control cultures had a higher level of DNA fragmentation at ~22% (Fig. 4). Sphingosine, sphinganine, and C_2-ceramide significantly increased DNA fragmentation to 37%, 47%, and 38%, respectively ($P < 0.05$). In contrast, DNA fragmentation in cells cultured with C_2-dihydroceramide was 24%, which was similar to controls (Fig. 4).

Flow Cytometric Analysis of Cell Cycle and Apoptotic HT-29 and HCT-116 Cells. The effects of sphingoid bases and ceramides at 35 μM for 24 hr on the populations of HT-29 and HCT-116 cells in each phase of the cell cycle were evaluated via flow cytometry (26, 27). As shown in a representative histogram in Figure 5, sphin-
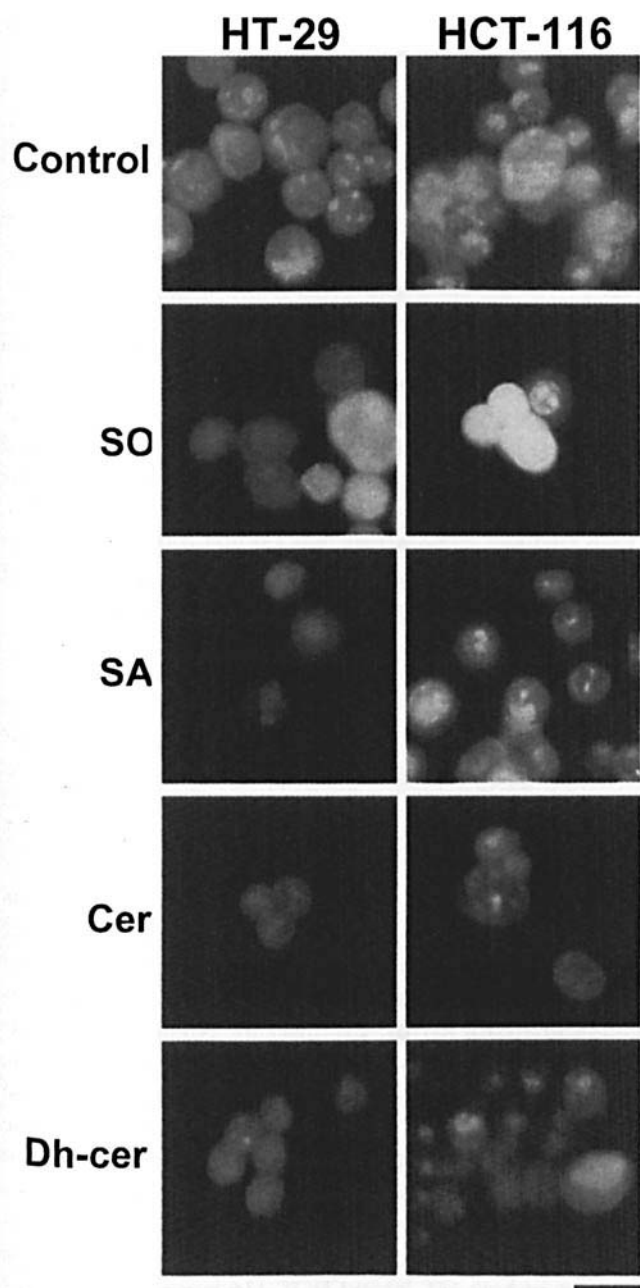
Figure 3. Effects of sphingoid bases and ceramides on chromatin and nuclear condensation in HT-29 and HCT-116 human colon cancer cells (photographs). Subconfluent cells were cultured in the absence (control) or presence of sphingosine (SO), sphinganine (SA), C_2-ceramide (Cer), and C_2-dihydroceramide (Dh-cer) at 20 μM for 24 hr. Cells were stained with acridine orange and ethidium bromide and were photographed under fluorescence microscope. Bar, 20 μm.
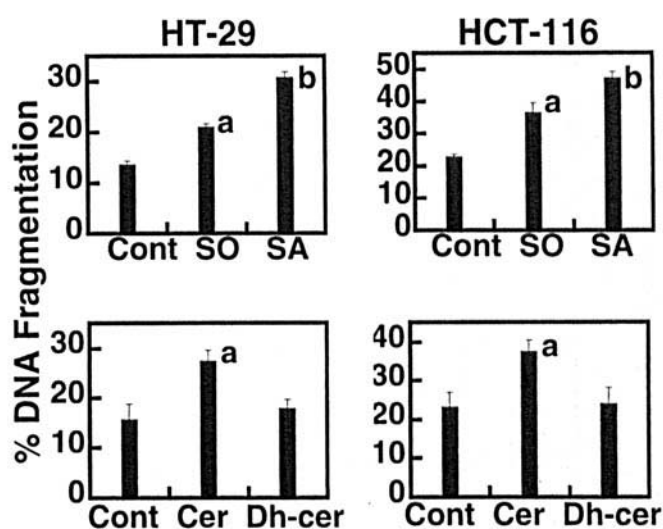

Figure 4. Effects of sphingoid bases and ceramides on fragmented low molecular DNA in HT-29 and HCT-116 human colon cancer cells. Subconfluent cells were cultured in the absence (control, Cont) or presence of sphingosine (SO), sphinganine (SA), C_2-ceramide (Cer), and C_2-dihydroceramide (Dh-cer) at 50 μM for 48 hr. Fragmented DNA were isolated and quantitated using the diphenylamine assay. Data are mean $\pm$ SEM ($n = 3$ for HT-29 cells; $n = 4$ for HCT-116 cells). Means with different letters are significantly different ($P < 0.05$).
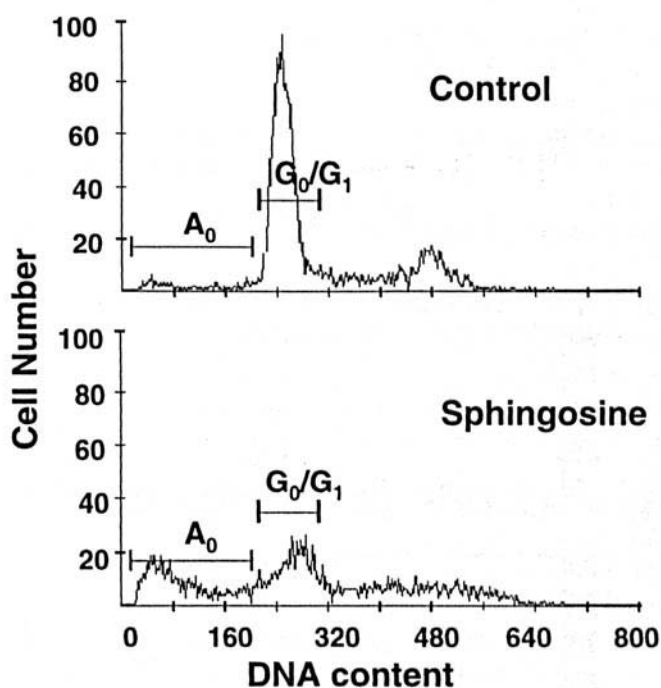

Figure 5. Sphingosine increases the number of A_0 (sub-G_0/G_1) cells indicative of apoptosis (one representative histogram). Subconfluent HCT-116 cells were cultured in the absence (control) or presence of sphingosine at 35 μM for 24 hr and stained with propidium iodide. The cell cycle and population were determined via flow cytometric analysis.

gosine potently reduced the number of HCT-116 cells with diploid DNA content (G_0/G_1 region) compared with control, and caused a corresponding increase in the number of cells with hypodiploid DNA content (sub-G_0/G_1 region) indicative of apoptosis (called A_0). To quantify the degree of apoptosis, cells in the A_0 peak were counted and expressed as a percentage of the total cell population. For HT-29 cells, only about 0.8% of the control cells were in the A_0 apoptotic peak, whereas sphingosine, sphinganine, and C_2-ceramide increased the number of apoptotic cells to 2%, 3%, and 8%,

respectively (Fig. 6). In comparison, HT-29 cells cultured with C_2-dihydroceramide were similar to control cultures having only ~1% in the A_0 apoptotic peak.

Similar but more profound effects were seen in HCT-116 cells. Control cultures had 1% to 5% of the cells in the
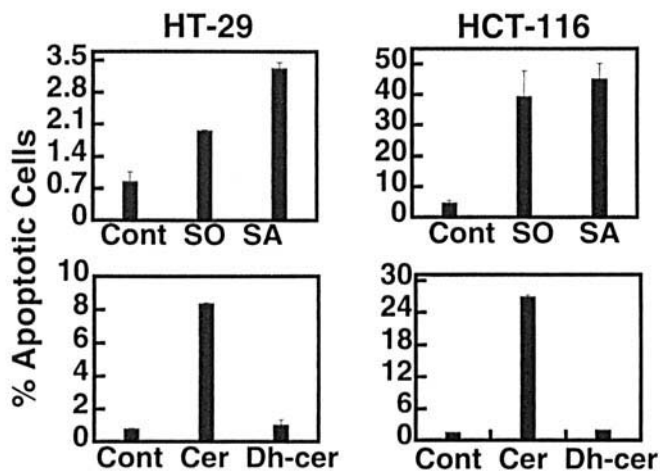
Figure 6. Effects of sphingoid bases and ceramides on apoptotic cell number in HT-29 and HCT-116 human colon cancer cells. Subconfluent cells were cultured in the absence (control, Cont) or presence of sphingosine (SO), sphinganine (SA), C_2-ceramide (Cer), and C_2-dihydroceramide (Dh-cer) at 35 µM for 24 hr. DNA was then stained with propidium iodide, the cell cycle was examined via flow cytometric analysis, and the percentage of cells in the A_0 (sub-G_0/G_1) region was estimated with the FCS express version 1.0 software. Data are mean ± SEM ($n = 2$).

A_0 apoptotic peak, whereas sphingosine, sphinganine, and C_2-ceramide increased the number of apoptotic cells to 39%, 45%, and 27%, respectively (Fig. 6). In contrast, only ~2% of the cells cultured with C_2-dihydroceramide were apoptotic, which was similar to control.

The percentages of HT-29 cells in G_0/G_1, S, and G_2/M (mean ± SEM) (Fig. 7) for each treatment were: control for sphingoid bases (65.9 ± 0.4, 25.6 ± 1.5, and 8.6 ± 1.1), sphingosine (41.2 ± 6.3, 45.7 ± 4.7, and 13.2 ± 1.7), sphin-
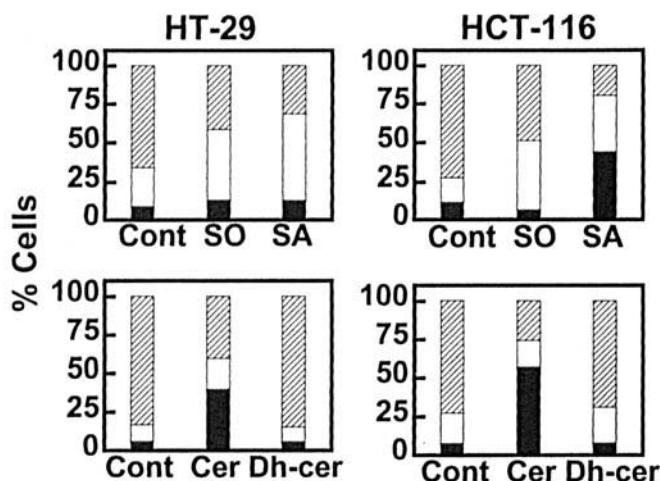

Figure 7. Effects of sphingoid bases and ceramides on cell cycle distribution of HT-29 and HCT-116 human colon cancer cells. Subconfluent cells were cultured in the absence (control, Cont) or presence of sphingosine (SO), sphinganine (SA), C_2-ceramide (Cer), and C_2-dihydroceramide (Dh-cer) at 35 µM for 24 hr. DNA was then stained with propidium iodide, the cell cycle was examined via flow cytometric analysis, and the percentage of cells in each stage of the cell cycle, including G_0/G_1 phase, ▨; S phase, □; and G_2/M phase, ■, was determined using the multi-cycle DNA content and cell cycle analysis software. The A_0 (sub-G_0/G_1) cell population was not included in calculation of cell population. Data are mean ± SEM ($n = 2$).

ganine (31.5 ± 0.6, 55.8 ± 0.8, and 12.7 ± 0.3), control for ceramides (83.2 ± 2.5, 11.0 ± 1.5, and 5.9 ± 1.0), C_2-ceramide (40.5 ± 6.2, 19.7 ± 2.7, and 39.9 ± 3.5), and C_2-dihydroceramide (84.8 ± 1.5, 9.4 ± 1.4, and 5.9 ± 0.2).

The percentages of HCT-116 cells in G_0/G_1, S, and G_2/M (mean ± SEM; Fig. 7) for each treatment were: control for sphingoid bases (72.3 ± 4.1, 16.3 ± 2.3, and 11.5 ± 1.8), sphingosine (48.8 ± 2.3, 45.0 ± 1.0, and 6.2 ± 1.3), sphinganine (19.9 ± 4.7, 36.3 ± 5.7, and 43.9 ± 10.3), control for ceramides (73.0 ± 1.0, 19.5 ± 0.5, and 7.6 ± 0.4), C_2-ceramide (25.8 ± 1.6, 17.2 ± 0.6, and 57.1 ± 2.1), and C_2-dihydroceramide (69.0 ± 4.0, 23.0 ± 1.0, and 8.0 ± 0.5).

Sphingosine and sphinganine caused a greater percentage of HT-29 and HCT-116 cells in the S phase compared with controls (Fig. 7). This increase in S phase cell population was accompanied by decreased G_0/G_1 cells (Fig. 7). These findings demonstrate that sphingosine and sphinganine arrested the cell cycle at the G_2/M phase and caused cell accumulation in the S phase.

Discussion

Complex dietary sphingolipids, including sphingomyelin and glycosphingolipids, have gained attention for their potential to protect against the development of colon cancer. These compounds reduce the number of aberrant colonic foci and the percentage of adenocarcinomas in chemically initiated mice (2–5) and they decrease the number of tumors in Min mice (6). The mechanism by which sphingolipids inhibit colon cancer is not clear; however, it is known that suppression of colon carcinogenesis by complex sphingolipids is not dependent on the complexity of the sphingolipid head groups (5, 6). This raises the possibility that their bioactive digestion products, ceramide and sphingoid bases, may mediate the inhibitory effects of sphingolipids. These molecules are putative second messengers in cells and have been shown to reduce proliferation and induce apoptosis in a variety of cancer cells (1, 7–9). This study demonstrates that both of the sphingoid bases sphingosine and sphinganine, as well as C_2-ceramide inhibit proliferation and cause the death of both HT-29 and HCT-116 human colon cancer cell lines at 10 to 50 µM. This concentration range of sphingoid bases and ceramide is similar to that previously shown to inhibit growth and/or cause differentiation or death in a variety of tumor cells (7–9, 28–34). The finding that a portion of the cell population survived the insult from sphingoid base and ceramide treatments and subsequently grew slowly between 24 and 48 hr is probably due to metabolism of these compounds either via incorporation into more complex sphingolipids or phosphorylation and degradation (35–37).

Presently, it is not clear whether colonic tissue is normally exposed to the concentrations of sphingoid bases and/or ceramide used in this study. Schmelz et al. (38) reported that ~88% of dietary sphingomyelin is digested and absorbed as ceramides and sphingoid bases in the small intestine, and small amounts of dietary sphingolipids appear in

lymph (39, 40) and serum (41). Therefore, colonic tissue could be exposed to ceramide and sphingoid bases via the circulation. Alternatively, ~12% of dietary sphingomyelin is not absorbed and passes directly into the colonic lumen (38) where it may be metabolized by colonic bacteria. This would provide a more direct route by which colonic tissues may be exposed to ceramide and sphingoid bases.

Another important finding of this study is that C_2-dihydroceramide, which lacks the 4,5-*trans* double bond present in C_2-ceramide, had no effect on the proliferation or death of either HT-29 or HCT-116 human colon cancer cells. This result establishes that the 4,5-*trans* double bond is necessary for the growth inhibitory and cytotoxic properties of C_2-ceramide in human colon cancer cells, but is not required by sphingoid bases. This is consistent with the results of previous studies which showed that short-chain ceramides caused apoptosis in many systems, whereas dihydroceramides lacking the 4,5-*trans* double bond had no biological effects (33, 42–47). In contrast, both sphingosine and sphinganine inhibit growth and induce apoptosis in a variety of cell lines and tumor xenografts (9, 32, 48, 49). Interestingly, previous studies have indicated that synthetic dihydrosphingomyelin (*N*-palmitoyldihydrosphingomyelin), which lacks the 4,5-*trans* double bond, was even more effective than synthetic sphingomyelin (*N*-palmitoyl-sphingomyelin) at reducing the number of aberrant colonic foci in CF-1 mice treated with DMH (4). Similarly in the present study, sphinganine (which lacks the 4,5-*trans* double bond) more potently increased DNA fragmentation than did sphingosine. Thus, complex sphingolipids may inhibit colon carcinogenesis via turnover to the free sphingoid bases (sphinganine and/or sphingosine) rather than to ceramide and dihydroceramide; however, from a practical perspective most complex sphingolipids present in food contain the 4,5-*trans* double bond (50). Jarvis *et al.* (49) showed that a combination of ceramide and a sublethal concentration of sphingosine or sphinganine was more effective at causing the death of two human myeloid leukemia cell lines, HL-60 and U-937, than ceramide alone. Therefore, another way in which complex sphingolipids may inhibit colon carcinogenesis is via turnover to a mixture of ceramide, sphingosine, and sphinganine.

The present study demonstrates that sphingosine, sphinganine, and C_2-ceramide kill HT-29 and HCT-116 human colon cancer cells by inducing apoptosis. These findings are consistent with those of Veldman *et al.* (8) who showed that C_2-ceramide induces apoptosis in HT-29 cells. Our findings underscore the possibility of utilizing sphingoid bases as well as ceramide or synthetic analogs as chemopreventive or chemotherapeutic agents for human colon cancer. Many chemotherapeutic agents have been shown to kill susceptible cells via apoptosis. For example, nonsteroidal anti-inflammatory drugs (NSAID) such as aspirin (51), sulindac (52, 53), piroxicam (53), and indomethacin (54) inhibit colon carcinogenesis via induction of apoptosis. Also, it is noteworthy that the anticarcinogenic ef-

fects of many chemotherapeutic agents are associated with activation of sphingomyelinase and generation of ceramide (and possibly sphingosine). Chan *et al.* (55) reported that sulindac, the most extensively investigated NSAID, induced apoptosis in HCT-116 and SW-480 human colon cancer cells by increasing the level of arachidonic acid, which stimulated conversion of sphingomyelin to ceramide. Chemotherapeutic agents that induce apoptosis appear to have the advantage of targeting individual cells without eliciting an inflammatory response in the surrounding normal tissue. Sphingosine may also have this property because it has previously been shown to induce apoptosis in neoplastically transformed human umbilical vein endothelial cells and rat mesangial cells (9) as well as human breast epithelial cells (Hong *et al.*, unpublished data), but not in their primary culture counterparts.

To our knowledge, this is the first study to demonstrate that sphingoid bases arrest the colon cancer cell cycle at the G_2/M phase and cause accumulation of cells in the S phase. Apoptosis triggered under these conditions is often found in response to suppression of DNA replication or DNA repair in drug-induced cell death and appears to involve inhibition of topoisomerase I or II (56). For example, DNA topoisomerase inhibitor I (camptothecin) or II (teniposide) induced apoptosis in HL-60 cells with S phase accumulation (56, 57). In addition, the anticancer drug 5-fluorouracil induced apoptosis by arresting the cell cycle at G_2 phase in human breast cancer grated in nude mice (58) and mitomycin C and etoposide induced apoptosis with G_2/M phase arrest and S phase accumulation in HCT-116 human colon cancer cells (59). Folate deficiency-induced apoptosis was found to coincide with a block at G_2/M and accumulation of cells in the S phase (60). Also, diallyl disulfide isolated from garlic (which is believed to reduce tumor incidence and suppress tumorigenesis) arrested HCT-15 cells in the G_2/M phase and increased the percentage of cells in the S phase (61, 62).

The mechanism by which sphingoid bases and ceramide induce apoptotic cell death is an area of active investigation, but is still not clearly understood. Though the present study did not examine targets of ceramide and sphingoid bases, there does not appear to be a role for *p53* as a mediator of ceramide or sphingoid base-induced apoptosis in HT-29 cells because these cells overexpress a mutated nonfunctional *p53* (12, 13). Furthermore, Dbaibo *et al.* (63) have shown that ceramide inhibits cell growth, arrests the cell cycle, and induces apoptosis, but does not increase *p53* expression in HL-60 and U937 human leukemia cells lacking functional *p53*. In addition, *Bax* (a pro-apoptotic member of the *Bcl-2* family genes) probably does not play a major role in ceramide-induced apoptosis in HT-29 and HCT-116 cells. Though HT-29 cells do not appear to have mutations in *Bax* (64) and HCT-116 cells are heterozygous for *Bax* (Bax +/+, +/−, −/−) (64, 65), Kim *et al.* (66) found that Bax protein expression did not change in C_2-ceramide-induced apoptosis of HT-29 cells, and Zhang *et al.* (65)

reported that C_2-ceramide-induced apoptosis was effected very little in HCT-116 cells carrying only *Bax* (–/–).

In summary, this study systematically evaluated the effects of the sphingoid bases, sphingosine and sphinganine, as well as C_2-ceramide and C_2-dihydroceramide on the growth, death, and cell cycle of HT-29 and HCT-116 human colon cancer cells. The sphingoid bases and C_2-ceramide were found to kill colon cancer cells by inducing apoptosis, whereas C_2-dihydroceramide was not effective. These data suggest that ceramide and the sphingoid bases have the potential to mediate the protective effects of more complex dietary sphingolipids and they raise the possibility that they themselves may be effective chemopreventive and chemotherapeutic agents for human colon cancer. Sphingoid bases appear to specifically arrest the cell cycle at G_2/M and cause accumulation of cells in the S phase similar to apoptosis induced by several other anticancer drugs.

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