Induction of Apoptosis by Sphingosine, Sphinganine, and C_2-Ceramide in Human Colon Cancer Cells, but not by C_2-Dihydroceramide

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Abstract. *Complex dietary sphingolipids such as sphingomyelin and glycosphingolipids have been reported to inhibit the development of colon cancer. This protective role may be the result of the conversion of complex sphingolipids to bioactive metabolites including sphingoid bases (sphingosine and sphinganine) and ceramide, which inhibit proliferation and stimulate apoptosis. In the current study, we evaluated the significance of the 4,5-trans double bond by comparing the effects of sphingosine and the cell permeable short-chain ceramide analog C_2-ceramide to those of sphinganine and C_2-dihydroceramide, which lack this structural feature. The effects of the sphingoid bases, C_2-ceramide, and C_2-dihydroceramide on apoptosis were determined by detecting 200-bp DNA ladders or hypo-diploid areas (sub-G_0/G_1), indicative of apoptosis, in HCT-116 human colon cancer cells. In addition, the effects of the sphingoid bases at an apoptotic concentration for 12 hours on cell cycle distribution were determined by flow cytometry. The results indicated that the sphingoid bases and C_2-ceramide induced apoptosis, whereas C_2-dihydroceramide had no effects. Sphingoid bases arrested the cell cycle at the G_2/M phase. The present study provides evidence that the 4,5-trans double bond is necessary for the apoptotic effect of C_2-ceramide, but not for that of sphingoid bases.*

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). Sphingolipids are found in all eukaryotic cell membranes, some prokaryotes, and also in a variety of foods including dairy and soy products (1, 2).

Sphingolipids have gained much attention for their potential to protect against the development of colon cancer (1). Complex dietary sphingolipids including sphingomyelin, dihydrosphingomyelin, glucosylceramide, lactosylceramide, and ganglioside GD_3 reduced aberrant colonic foci in CF1 mice treated with 1,2-dimethylhydrazine (DMH) (3-8) and 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 (8, 9).

This protective role of sphingolipids against colon carcinogenesis may be the result of the conversion of complex sphingolipids to bioactive metabolites including sphingoid bases (sphingosine and sphinganine) and ceramide, which inhibit proliferation and induce apoptosis (programmed cell death) in various types of cancer cell (10, 11).

To date, most studies examining sphingolipids have focused on the proapoptotic actions of ceramide (acylated form of sphingoid bases) and few studies have examined the actions of sphingoid bases. Furthermore, it is uncertain whether the sphingosine or sphinganine backbones of ceramides mediate the antiproliferative and proapoptotic activities of ceramide on cancer cells (12-15). In the current study, we evaluated the significance of the 4,5-*trans* double bond by comparing the effects of sphingosine and the cell permeable ceramide analog C_2-ceramide to those of sphinganine and C_2-dihydroceramide, which lack this structural feature. We also investigated the effects of sphingoid bases, C_2-ceramide and C_2-dihydroceramide on apoptosis and cell cycle distribution in HCT-116 human colon cancer cells.

Materials and Methods

Cell culture. The HCT-116 human colon cancer cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). Stock cultures of HT-29 and HCT-116 cells were

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cultured in 100 mm dishes (Corning, Cambridge, MA, USA) containing Dulbeco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 3.5 g glucose/l and 2.5 ml penicillin-streptomycin/l (Invitrogen) at 37˚C and 5% CO_2. All experiments were performed using cells with passages less than 20.

Sphingolipids treatments. Sphingoid bases (sphingosine and sphinganine), C_2-ceramide, and C_2-dihydroceramide were purchased from Matreya (Pleasant Gap, PA, USA) and were prepared as described in our previous study (11). C_2-ceramide and C_2-dihydroceramide are cell permeable short chain analogs of naturally occurring ceramide and dihydroceramide, respectively. The HCT-116 cells were seeded at a density of 2×10^5 cells/ml (*i.e.* $1.8\times10^4/cm^2$ growth area) in 100 mm dishes and cultured with 5 ml of DMEM with 10% FBS for 24 h to ensure that the cells were in log phase before treatment. The medium was then replaced with DMEM supplemented with 1% FBS and the cells were treated with or without sphingolipids at 20 or 35 μM for 12 or 24 hours.

Analysis of internucleosomal DNA fragmentation by gel elctrophoresis. Fragmented DNA was detected using a Genomix cells small scale kit (Talent, Trieste, Italy). The procedure was based on that of Goruppi *et al*. (16). The cells were harvested in 150 μl of phosphate-buffered saline (PBS). The cells were then lysed and the aqueous phase was separated using chloroform, acidification solution, and gel barriers. The cells were precipitated and centrifuged and the liquid phase was removed. The DNA pellet was resuspended in distilled water followed by electrophoresis in a 2% agarose gel at 100 V for 70-90 min. The DNA bands stained with ethidium bromide were photographed under UV light.

Flow cytometric analysis of cell cycle and population. The cells were seeded at a density of 2×10^5 cells/ml and cultured with sphingolipids at 35 μM for 12 h. Flow cytometric analysis was performed as described in our previous study (11).

Results

Sphingoid bases and C_2-ceramide induce apoptosis, while C_2-dihydroceramide has no effects. The effects of sphingoid bases (sphingosine and sphinganine) at 35 μM for 12 hours on apoptosis in HCT-116 cells were evaluated by determining the hypo-diploid DNA content (sub-G_0/G_1 region, called A_0), indicative of apoptosis, *via* flow cytometry. The cells in the A_0 peak were counted and expressed as a percentage of the total cell population. The control cultures had approximately 1.6% of cells in the A_0 apoptotic peak. Sphingosine and sphinganine increased the A_0 apoptotic peak to 7% and 12%, respectively (Figure 1).

The effects of C_2-ceramide and C_2-dihydroceramide treatment at 20 μM for 24 hours on apoptosis were determined by analyzing internucleosomal DNA fragmentation (Figure 2). C_2-ceramide generated 200-bp DNA ladders, which are indicative of apoptosis, whereas C_2-dihydroceramide did not. This indicates that C_2-ceramide induces apoptosis, while C_2-dihydroceramide has no effects on HCT-116 human colon cancer cells.

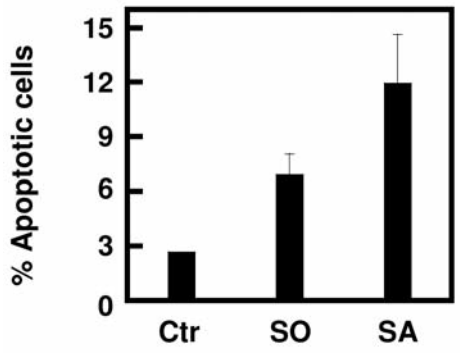

Figure 1. *Sphingoid bases increase the apoptotic cell number (A_0) in HCT-116 human colon cancer cells. Subconfluent cells were cultured in the absence (control, Ctr) or presence of sphingosine (SO) and sphinganine (SA) at 35 μM for 12 h. The DNA was then stained with propidium iodide, the cell cycle was examined via flow cytometric analysis, and the percentage of cells in the A_0 (sub-G_0/G_1) region was estimated with FCS express version 1.0 software. Representative data are mean±SEM (n=2). Where an error bar is not shown, it lies within the dimensions of the symbol.*

Sphingoid bases arrest the cell cycle at the G_2/M phase. The effects of sphingoid bases (sphingosine and sphinganine) applied at 35 μM for 12 hours on cell cycle distribution in HCT-116 cells were determined by flow cytometry. The percentages of HCT-116 cells in G_0/G_1, S, and G_2/M phases (Figure 3) for each treatment were as follows (mean±SEM): control: 47.7±4.5, 44.2±3.1, 8.1±1.8; sphingosine: 14.8±2.7, 59.3±2.5, 26.0±0.2; and sphinganine: 20.9±2.0, 45.6±1.2, 33.5±0.8. Sphingosine and sphinganine caused a much greater percentage of HCT-116 cells to be in the G_2/M phase compared to the control. This increase in G_2/M phase cell population was initially accompanied by a decrease in G_0/G_1 phase cells (Figure 3). This finding demonstrates that sphingoid bases arrested the cell cycle at the G2/M phase.

Discussion

In both our previous study and the current study, we demonstrated that sphingosine, sphinganine, and C_2-ceramide inhibited growth and arrested the cell cycle at G_2/M phase and induced apoptosis in HT-29 and HCT-116 human colon cancer cells, whereas, C_2-dihydroceramide had no effect (11). In our previous study, we demonstrated that the treatment with sphingoid bases and C_2-ceramide at 35 μM for 24 h induced apoptosis. In the current study, sphingoid bases applied at 35 μM for 12 h also induced apoptosis but the extent of apoptosis was slightly smaller than that of the sphingoid bases applied for 24 h. In our previous study, the control had an A_0 peak of ~4.6%, while using sphingosine and sphinganine for 24 h increased the A_0 apoptotic peak to 39 and 45%, respectively (11). In the current study, the

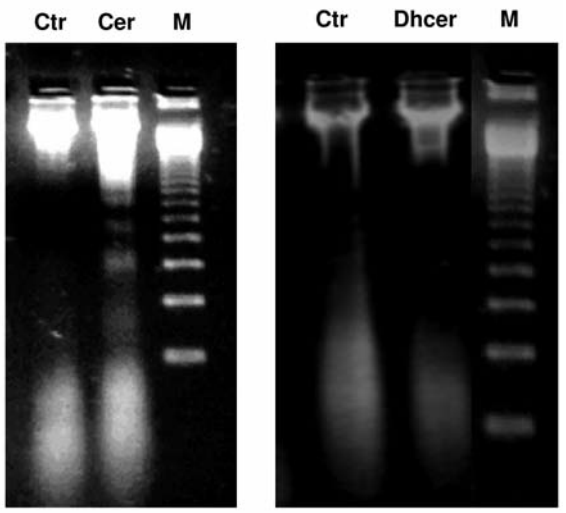

Figure 2. *C2-ceramide generates DNA fragmentation, indicative of apoptosis, in HCT-116 human colon cancer cells, whereas C_2-dihydroceramide has no effects. Subconfluent HCT-116 cells were cultured without sphingolipids (control, Ctr), or with C_2-ceramide (Cer), or C_2-dihydroceramide (Dhcer) at 20 μM for 24 h. The DNA was extracted and analyzed using gel electrophoresis on a 2% agarose gel at 100 V. M, DNA marker (123 bp).*

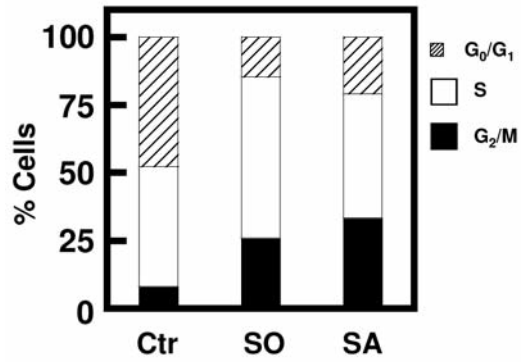

Figure 3. *Sphingoid bases arrest the cell cycle at G_2/M phase in HCT-116 human colon cancer cells. Subconfluent cells were cultured in the absence (control, Ctr) or presence of sphingosine (SO) and sphinganine (SA) at 35 μM for 12 h. The DNA was then stained with propidium iodide, the cell cycle was examined via flow cytometric analysis, and the percentage of cells in each stage of the cell cycle 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 the calculation of the cell population. Representative data are the mean±SEM (n=2).*

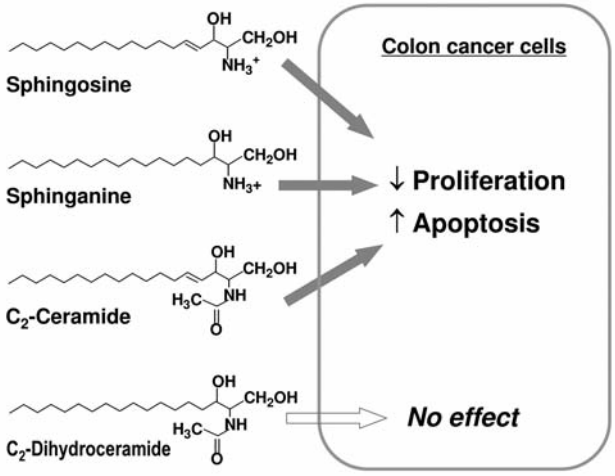

Figure 4. *Sphingosine, sphinganine and C_2-ceramide inhibit growth and induce apoptosis, whereas C_2-dihydroceramide has no effect in human colon cancer cells.*

control cultures had an A_0 peak of about 1.6%, while in the cells treated with sphingoid bases for 12 h the A_0 apoptotic peak increased to 12%. This suggests that sphingoid bases at the same growth-inhibitory concentration induce apoptosis in a time-dependent manner. In the current study, C_2-ceramide at 20 μM, a lower concentration than we tested previously, used for 24 h also induced apoptosis, whereas C_2-dihydroceramide had no effects.

Both our previous and current results establish that the 4,5-*trans* double bond is necessary for antiproliferative and proapoptotic properties of C_2-ceramide in human colon cancer cells, but is not required for sphingoid bases to exert those effects. This is consistent with the results of previous studies which showed that short-chain ceramides caused apoptosis, while dihydroceramides lacking the 4,5-*trans* double bond had no biological effects (17-23). In contrast, both sphingosine and sphinganine inhibit growth and induce apoptosis in various cancer cell lines and tumor xenografts (10, 24-26). This led us to suggest that the inhibitory effects of both dihydrosphingomyelin, which lacks the 4,5-*trans* double bond, and sphingomyelin, which has the 4,5-*trans* double bond, on colon carcinogenesis may be due to turnover to free sphingoid bases (sphinganine and sphingosine) rather than to ceramide and dihydroceramide.

However, the mechanisms for the lack of biological activity of C_2-dihydroceramide remain unclear. Future study can determine the binding efficiency of C_2-ceramide and C_2-dihydroceramide to cellular membranes and the uptake of the ceramides into cells, as well as the cellular concentrations of ceramide, dihydroceramide, or free sphingoid bases. Based on a previous *in vitro* study, a possible mechanism for the lack of biological activity of C_2-dihydroceramide may be due to the inability of C_2-dihydroceramide to activate ceramide-activated protein phosphatase (27).

In summary, the treatment of HCT-116 human colon cancer cells with sphingoid bases and C_2-ceramide for 12 or

24 h induced apoptosis, whereas C_2-dihydroceramide had no effects. The present study provides evidence that the 4,5-*trans* double bond is necessary for the apoptotic effects of C_2-ceramide, but not for those of sphingoid bases (Figure 4).

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