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SPHINGOLIPIDS IN FOODS AND DIFFERENTIAL SENSITIVITY OF HUMAN COLON CANCER CELLS TO SPHINGOID BASES AND CERAMIDES

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ABSTRACT

SPHINGOLIPIDS IN FOODS AND DIFFERENTIAL SENSITIVITY OF HUMAN COLON CANCER CELLS TO SPHINGOID BASES AND CERAMIDES

By

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Complex sphingolipids have been shown to protect against development of colon cancer. Sphingoid bases and ceramides are bioactive metabolites of sphingomyelin and other more complex sphingolipids found in foods. The purpose of the present study was to quantitate sphingolipids in some common foods and investigate the effects of sphingoid bases and ceramides on growth and death of HT-29 and HCT-116 human colon cancer cells. The concentrations of total sphingolipids and free sphingoid bases (nmol/g of dry weight) were approximately: nonfat dry milk (203, 146); yogurt (138, 1.2); Swiss cheese (167, 6.5); full fat soy flakes (609, 2.6); soy flour (610, 1.6); isolated soy protein (210, 2.8). Most sphingolipids in foods were present as complex sphingolipids and sphingosine was the predominant sphingoid base backbone of total sphingolipids in foods. Sphingosine, sphinganine, and C_2-ceramide inhibited growth and caused death of HT-29 and HCT-116 cells in concentration- and time-dependent manners; whereas, C_2-dihydroceramide had no effect suggesting that the 4,5-trans double bond was necessary for the inhibitory effect of ceramide. Sphingosine and sphinganine killed cells by inducing apoptosis; whereas, C_2-ceramide did not induce apoptosis. The results indicate that sphingolipids are significant constituents of soy fractions and dairy products and that the colonic concentrations of sphingoid bases and/or ceramide which may be achieved after consumption of the foods are sufficient to inhibit growth and cause death of human colon cancer cells.

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I dedicate this fruit of my sweat to my parent who gave me a life, my older brothers, relatives, special friends, and God who created the world.

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I. LITERATURE REVIEW

A. Colon cancer

1. Colon cancer incidence. Colon cancer is the second leading cause of cancer mortality in the United States (American Cancer Society, 1996). In the world, colon cancer is the fourth most common cancer mortality (World Cancer Research Fund, 1997). North America, Europe and Australia have high incidence of colon cancer; whereas, Central and South America, Asia and Africa are areas of low incidence. Men and women have a similar rate of incidence of colon cancer (World Cancer Research Fund, 1997).

2. Colon cancer etiology. The adenoma-carcinoma hypothesis proposed by Hill *et al* (1978) is now a widely accepted description of the pathogenesis of colorectal cancer. The target cells of colon carcinogenesis are crypt epithelial cells (Hill *et al.*, 1978). According to this model, the initial colorectal lesion arises as a benign adenomatous polyp that later undergoes further disorganization of cellular and tissue phenotype. Bird *et al* (1987) suggested that hyperproliferation of the upper crypt cells leads to the formation of aberrant crypt foci and microadenomas. Increased proliferation and decreased differentiation of colonic epithelial cells are considered to be biomarkers for an increased risk for developing colon cancer (Lipkin, 1990). A molecular model for the adenoma-carcinoma sequence can be described by the multi-step process in which cells accumulate alterations of multiple genes that control cell growth and differentiation, resulting in the neoplastic phenotype (Vogelstein *et al.*, 1989, Fearon *et al.*, 1990, Fearon and Vogelstein, 1990, Kinzler *et al.*, 1991). The genes involved in colon

carcinogenesis are: mutations or loss of the *APC* gene (a tumor-suppressor gene) (Aaltonen *et al.*, 1993), mutation of *K-ras* (a proto-oncogene) and early disorganization of DNA methylation (Feinberg and Vogelstein, 1983) and late loss of *p53* (a tumor-suppressor gene). People having familial adenomatous polyposis possess a mutation in the *APC* gene and carry an almost 100 percent risk of colon adenocarcinoma (Aaltonen *et al.*, 1993). Hereditary nonpolyposis colorectal cancer is a syndrome which is not easily distinguished from sporadic polyposis and cancer on physical examination. This syndrome accounts for a larger proportion of colon cancer cases than familial adenomatous polyposis (Lynch and Lynch, 1985). DNA hypomethylation is an early event in colon carcinogenesis (Feinberg and Vogelstein, 1983). DNA methylation is genetically controlled and the expression of the methyl transferase gene seems to be increased in the normal mucosa of cancer patients and further increased in polyp and cancer tissue (El-Deiry *et al.*, 1991). Several animal studies showed that isothiocyanates, which mainly are present in cruciferous vegetables, inhibited both carcinogenesis and DNA methylation (Wattenberg, 1977, Wattenberg, 1987, Steinmetz and Potter, 1991a, & Steinmetz and Potter, 1991b). Although these observations are contradictory, both hypo- and hypermethylation of DNA are considered to be hallmarks of the early stages of the carcinogenesis.

3. Colon cancer and diet. Epidemiological studies of diet, nutrient intake and colon cancer indicate that meat consumption which reflects fat and protein intake is associated with high incidence of colon cancer (Wynder and Shigematsu, 1967, Wynder *et al.*, 1969, Drasar and Irving, 1973, Haenszel *et al.*, 1973, Bjelke, 1973, Armstrong and Doll, 1975, Hirayama, 1981, Heaton, 1982, Phillips and Snowdon, 1985, Potter, 1989)

and fruit and vegetable consumption reflecting fiber intake is associated with a protective effect against development of colon cancer (Burkitt, 1969, Manousos *et al.*, 1983, Stubs, 1985, Trock *et al.*, 1990, Potter, 1990, Steinmetz and Potter, 1991a, & Steinmetz and Potter, 1991b). Bile acid metabolism and volatile fatty acids might account for the association of fat and fiber consumption with colon cancer. Fat increases bile acid production and then bowel mucosa is more exposed to toxic bile acids (Hill, 1971). In contrast, fiber binds bile acids, reduces transit time, increases stool bulk, and increases bile acids fermentation to non-toxic volatile fatty acids (Stephen and Cummings, 1983, Cummings, 1983).

Other factors which might affect risk of colon cancer include physical activity (Garabrant *et al.*, 1984, Gerhardsson *et al.*, 1986, Slattery *et al.*, 1988), family history (Burt *et al.*, 1985, Bufill, 1990), and alcohol consumption (Potter *et al.*, 1982, Tuyns *et al.*, 1988, Longnecker, 1990, Choi and Kahyo, 1991). Lee *et al* (1991) showed that individuals with high levels of physical activity were at the low risk for developing colon cancer (Garabrant *et al.*, 1984, Gerhardsson *et al.*, 1986, Slattery *et al.*, 1988). A family history of colon cancer is related with increased risk of colon cancer (Burt *et al.*, 1985, Bufill, 1990). Although some studies showed that alcohol consumption (mostly beer) was related with increased risk of colon cancer, cautious interpretation of these results is recommended (Potter *et al.*, 1982, Tuyns *et al.*, 1988, Longnecker, 1990, Choi and Kahyo, 1991).

Epidemiological studies suggest that dietary fat and specific dietary fatty acids are associated with colon cancer (Potter *et al.*, 1993); however, this association is controversial since dietary fat is highly correlated with total energy intake. Slattery *et al*

(1997) evaluated these potential associations using detailed dietary intake data collected in a population-based study of 1,993 colon cancer cases and 2,410 controls in three areas of the United States. In this study population, fats added in the preparation of foods such as fried foods or bakery products or fats added to other foods at the table were responsible for one-third of the total diet fat intake. Interestingly, neither total dietary fat nor specific fatty acids were associated with risk of colon cancer after adjusting for total energy intake, physical activity, and body size. However, fats from food preparation were associated with increased risk of colon cancer among older women, while fats from foods themselves or from additions to other foods were not. Taken together, it seems that the percentage of energy from fat in the diet is not a major indicator of colon cancer risk.

Several investigations suggested that soy consumption may contribute to the lower rates of breast, prostate, and colon cancer in China and Japan (Setchell *et al.*, 1984, Barnes *et al*, 1990, Adlercreutz, 1990). The isoflavone genistein, which is rich in soybeans and considered as one of the most significant plant estrogens, has been reported to protect against development of colon carcinogenesis (Akiyama *et al.*, 1987, Ogawara *et al.*, 1989, Teraoka *et al.*, 1989, Bourquin *et al.*, 1996, Thiagarajan *et al.*, submitted).

Other epidemiological studies indicate that regular consumption of fermented milk products such as yogurt may be protective against some forms of cancer (Peters *et al.*, 1992, Kampman *et al.*, 1994). Some lactic acid bacteria present in fermented milk, especially *Lactobacilli*, are components of intestinal microflora (Bianchi Salvadori, 1986) and have protective effects against pathogenic microorganisms (Mutai and Tanaka, 1987). For example, a dietary supplement of *Lactobacillus acidophilus* reduced the incidence of 1,2-dimethylhydrazine-induced colon cancer in F344 rats (Goldin and

Gorbach, 1980). Also, epidemiological studies have reported that, despite the high fat intake in Finland, colon cancer incidence is lower than in other countries (Malhorta, 1977, International Agency for Research on Cancer, 1983). This may be due to Finland's high consumption of milk, yogurt, and other dairy products. Furthermore, yogurt fractions obtained by membrane dialysis on cultured mammalian intestinal cells decreased cell proliferation in both IEC-6 and Caco-2 cells and reduced the number of IEC-6 cells in the initial growth phase (Ganjam *et al.*, 1997).

B. Dietary sphingolipids might reduce colon carcinogenesis.

1. Sphingolipids regulate cell behavior. Sphingolipids include ceramides, sphingomyelin, cerebrosides, sulfatides, and gangliosides, all of which are elaborations of a long-chain (sphingoid) base, the most common of which is an 18-carbon compound termed sphingosine (Figure 1). Sphingosine (trans-4-sphingenine) and sphinganine (without the 4,5-trans double bond) are the most prevalent free long-chain bases of most mammalian tissues (Merrill, 1991). Except for sphingoid bases, ceramides, and sphingomyelin, all other sphingolipids are designated glycosphingolipids since they contain carbohydrate head groups. Glycosphingolipids include neutral glycosphingolipids and acidic glycosphingolipids. Neutral glycosphingolipids contain from one (cerebroside) to 20 or more glucose units (Makita and Taniguchi, 1985, Hakomori, 1983). Acidic glycosphingolipids contain one or more sialic acid residues (gangliosides) or monoester groups (sulfatides) (Wiegandt, 1985).

Sphingolipids are located mainly in plasma membranes and related organelles including the Golgi apparatus, endosomes, and lysosomes which are functionally

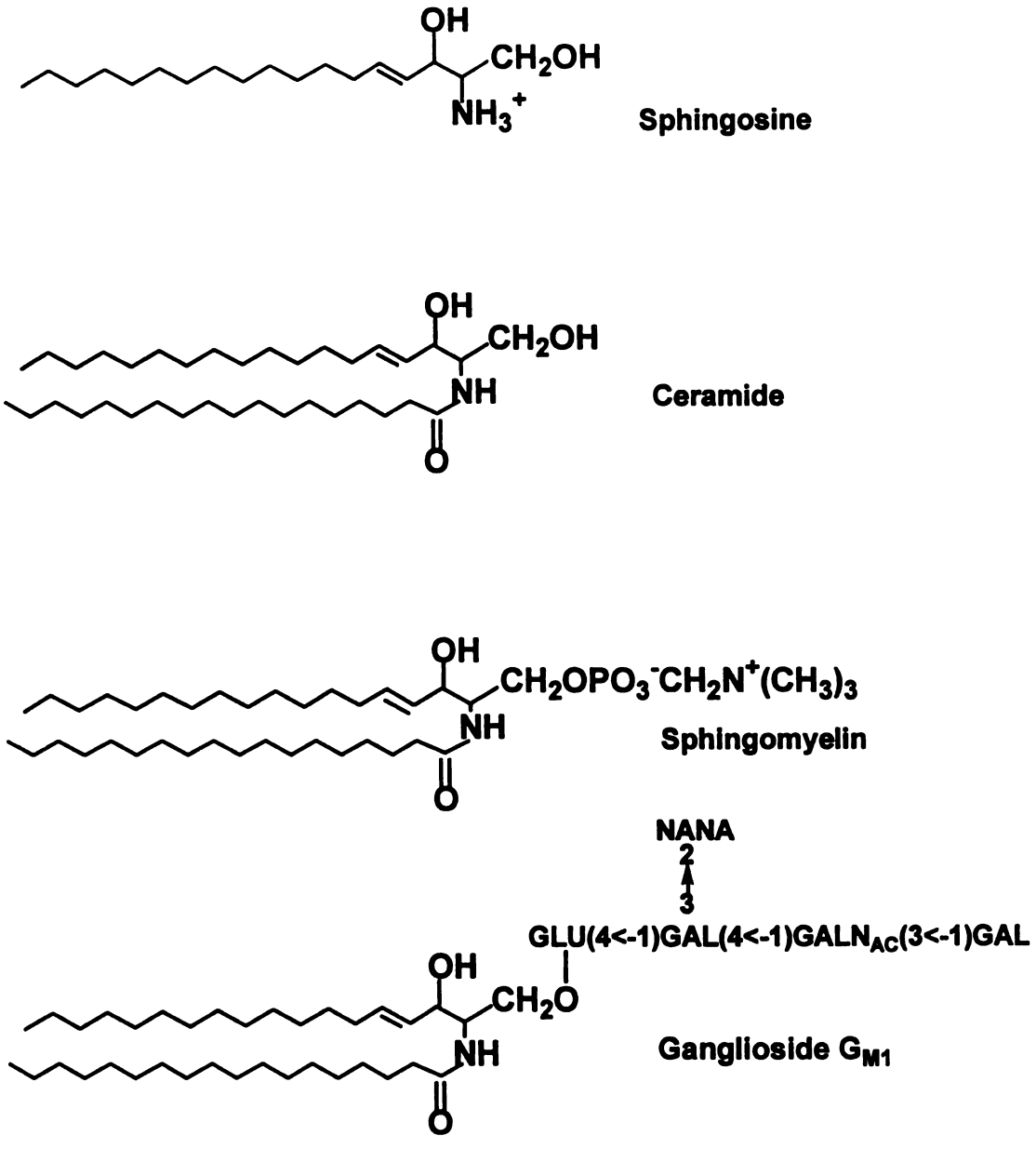

Figure 1. Structures of sphingolipids. Abbreviations used are: NANA, N-acetyl neuraminic acid; GAL, galactose; N_{AC}, N-acetyl; GLU, glucose.

associated with cellular responses to external agents such as growth factors, cytokines, extracellular matrix proteins, neighboring cells and microbial toxins and receptors (Merrill *et al.*, 1995b). Sphingolipids can regulate cell behavior at the surface of the cell, where they bind to extracellular ligands, such as bacterial toxins, lectins from neighboring cells, and antibodies. For instance, ganglioside G_{M1} binds to cholera toxin and mediates cholera toxin's effects on cells by activating adenylate cyclase (Fishman, 1982) and gangliosides G_{D1a}, G_{T1b}, and G_{Q1b} function as natural receptors for Sendai virus in host cells (Markwell *et al.*, 1981). In addition, carbohydrate sequences present in gangliosides are recognized by monoclonal antibodies raised against murine teratocarcinomas (Solter and Knowles, 1978, Gooi *et al.*, 1981, Kannagi *et al.*, 1982), carcinomas of the pancreas, lung, colon and stomach (Brockhaus *et al.*, 1982) and myeloid leukemia cells and granulocytes (Skubitz *et al.*, 1983, Urdal *et al.*, 1983, Magnani *et al.*, 1984). Sphingolipids might act to form a classical receptor-ligand interaction, or to define regions of the membrane with surface characteristics that aid in receptor binding and responses, internalization, recycling, or in anchoring proteins to the cell surface (Merrill *et al.*, 1993). An example of a sphingolipid acting at the surface of the cell is the interaction between ganglioside G_{M3} and epidermal growth factor receptor (Bremer *et al.*, 1986, Hanai *et al.*, 1988a, Hanai *et al.*, 1988b). Ganglioside G_{M3} inhibits epidermal growth factor-stimulated growth of human epidermoid carcinoma cells KB and A431. The mechanism involves inhibition by ganglioside G_{M3} of epidermal growth factor-stimulated phosphorylation of the epidermal growth factor receptors (Bremer *et al.*, 1986).

Sphingolipids can regulate cell behavior by changes that take place in the plasma membrane and/or endosomes, where complex sphingolipids and their turnover products

can stimulate or inhibit receptor kinases, responses of G proteins, protein kinases, protein phosphatases, and ion transporters (Merrill, 1991). Some sphingolipid hydrolysis products could influence both the external and internal leaflets of the plasma membrane. For example, Hope and Cullis (1987) showed that ceramides are relatively nonpolar and can cross membranes readily. In addition, sphingosine seems to undergo rapid movement across membranes in the neutral form and the hydroxyls at position 2 of the sphingoid base backbone gives a pKa near physiological pH (Merrill *et al.*, 1989).

Sphingolipids can regulate cell behavior at intracellular areas that are sensitive to products of sphingolipid turnover. Some sphingolipids, such as lysosphingolipids, *N*-deacylation products of sphingolipids, and free long-chain (sphingoid) bases, can move rapidly among membranes and might affect targets at sites distant from their locations of formation. For example, sphingosine might be liberated in the plasma membrane but affect protein kinase C in the nucleus (Hannun *et al.*, 1986a, Hannun *et al.*, 1986b). Sphingolipids are also able to be converted to other bioactive compounds, such as ceramides, ceramide 1-phosphate, sphingoid bases, sphingosine 1-phosphate, *N*-methylated sphingosines, and lysosphigolipids which can affect multiple intracellular targets (Zhang *et al.*, 1991, Merrill *et al.*, 1993).

2. Milk sphingomyelin might prevent the progression of adenomas to adenocarcinomas in colon cancer. Recently, sphingolipids have emerged as another component of the diet which may help to protect against development of colon carcinogenesis. Using rodents, Schmelz *et al* (1994) showed that about 12% of dietary sphingolmyelin passes through the small intestine to the colon. This finding raised the possibility that consumption of sphingomyelin may provide bioactive sphingolipid

metabolites such as ceramide and sphingoid bases which could inhibit the development of colon cancer (Figure 2 and Figure 3). This hypothesis was tested in initiation-promotion studies conducted by Merrill's group using sphingomyelin isolated from nonfat dry milk (Dillehay *et al.*, 1994, Schmelz *et al.*, 1996). The results showed that sphingomyelin at 0.05% of the diet inhibited formation of aberrant colonic foci in CF-1 mice treated with 1,2-dimethylhydrazine (Dillehay *et al.*, 1994). A subsequent longer term (34 weeks) study has shown that sphingomyelin at 0.1% of the diet does not reduce the number of tumors but causes a higher percentage of adenomas and lower percentage of the more advanced adenocarcinomas (Schmelz *et al.*, 1996). Also, the potential of synthetic sphingomyelins with saturated or unsaturated sphingoid base backbones to suppress the number of aberrant colonic foci was investigated using CF-1 mice treated with 1,2-dimethylhydrazine (Schmelz *et al.*, 1997). In this study, the reduction of the number of aberrant colonic foci by synthetic dihydrosphingomyelin (*N*-palmitoyldihydrosphingomyelin) (70%, $p < 0.0001$) was significantly greater than by synthetic sphingomyelin (*N*-palmitoylsphingomyelin) (52%, $p = 0.002$) and milk sphingomyelin (54%, $p = 0.002$). This indicates that the 4,5-trans double bond is not required for the suppression of colon carcinogenesis since synthetic dihydrosphingomyelin, which lacks the 4,5-trans double bond of the sphingoid base backbone, efficiently reduced the number of aberrant colonic foci (Schmelz *et al.*, 1997).

3. Ceramide and sphingosine may mediate the protective effect of sphingomyelin against colon cancer. The ability of dietary sphingomyelin to reduce colon carcinogenesis may be the result of turnover of sphingomyelin to bioactive metabolites such as ceramides and sphingoid bases which play important roles in signal

transduction and cell regulation (Figure 2 and Figure 3). Extracellular agonists, such as certain cytokines, growth factors, and hormones, stimulate their cell surface receptors to activate a sphingomyelinase which cleaves sphingomyelin to generate cellular ceramide (Figure 2). For example, 1α,25-dihydroxyvitamin D_3 (Okazaki *et al.*, 1989), tumor necrosis factor alpha (TNF-α) (Kim *et al.*, 1991, Mathias *et al.*, 1991), and γ–interferon (Kim *et al.*, 1991, Dressler *et al.*, 1992), which are inducers of differentiation of HL-60 human leukemia cells, cause hydrolysis of sphingomyelin to form ceramide. In addition, interleukin-1 (Ballou *et al.*, 1992, Mathias *et al.*, 1993), dexamethasone (Ramachandran *et al.*, 1990), complement components (Niculescu *et al.*, 1993), fungal macrolide brefeldin A (Linardic *et al.*, 1992), and β-sitosterol, the main phytosterol in the diet (Awad *et al.*, 1998), were found to induce hydrolysis of sphingomyelin in other cell lines.

C_2-ceramide and other short-chain water-soluble analogues of ceramide induced differentiation in HL-60 leukemia cells which mimicked the effects of 1α,25-dihydroxyvitamin D_3, tumor necrosis factor alpha (TNF-α), and γ-interferon on HL-60 cells (Okazaki *et al.*, 1990). Treatment of U937 human myeloid leukemia cells with TNF-α caused sphingomyelin hydrolysis and resulted in elevation of level of ceramide. In addition, ceramide analogs or TNF-α caused internucleosomal DNA fragmentation, a hallmark of apoptosis in myeloid and lymphoid cells (Obeid *et al.*, 1993). Unlike tissue necrosis, which occurs in response to severe insults and injury to cells, apoptosis involves an orderly breakdown of cells. Apoptosis is characterized with chromatin condensation followed by DNA fragmentation via the activation of an endonuclease, cytoplasmic blebbing, and condensation, and finally disintegration into dense particles called

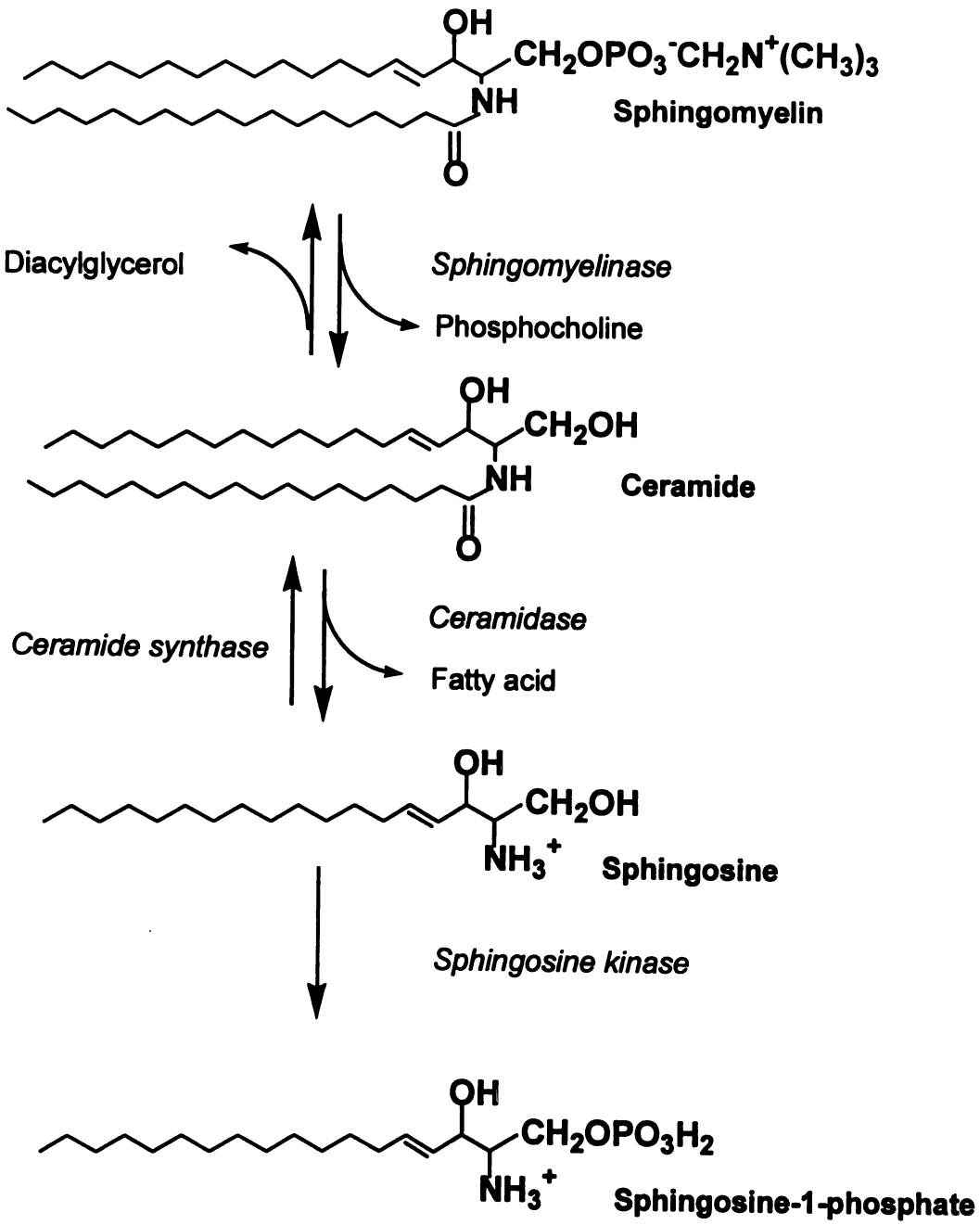

Figure 2. Bioactive products of sphingolipid hydrolysis.

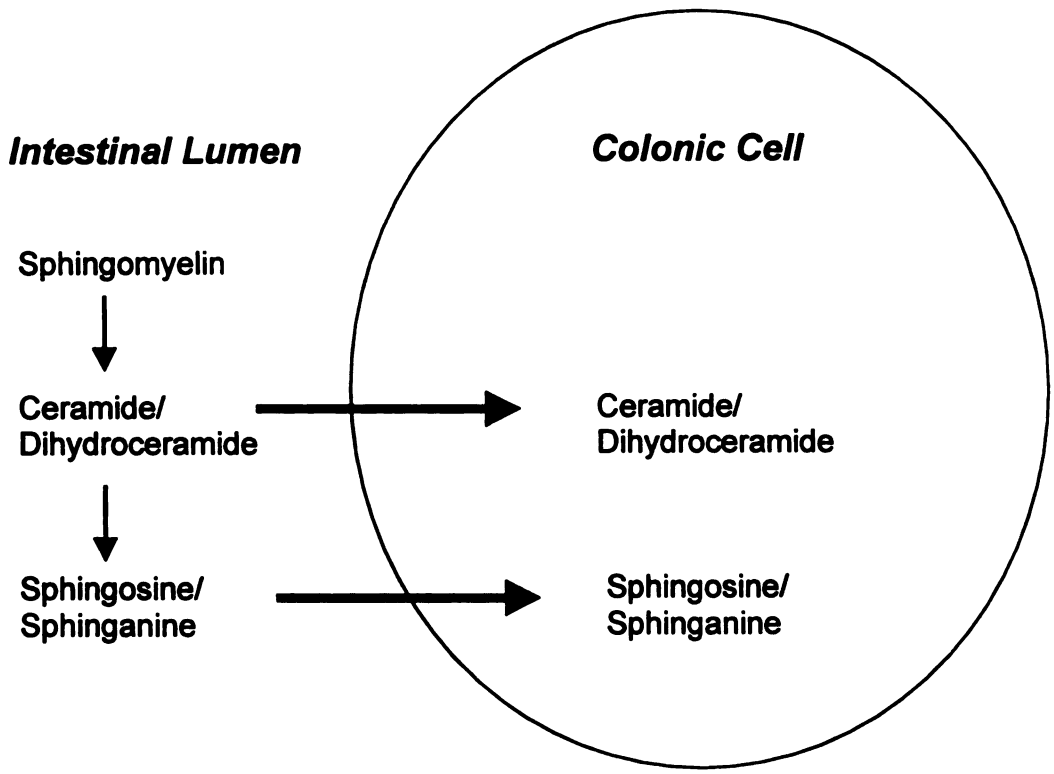

Figure 3. Dietary sphingomyelin and its turnover products can reach the colon.

apoptotic bodies (Michaelson, 1991). Ceramide serves as a second messenger for the action of extracellular agonists by transmitting the signal to the nucleus through multiple downstream targets such as protein kinase C (zeta isoform), ceramide-activated protein phosphatase (CAPP), and ceramide-activated protein kinase. For example, ceramide induced early down-regulation of *c-myc* protooncogene (Kim *et al.*, 1991). Ceramide activated the nuclear factor *kappa* B (NF-*k*B) in permeabilized (Schutze *et al.*, 1992) but not in intact Jurkat T cells (Dbaibo *et al.*, 1993). NF-*k*B (Schutze *et al.*, 1992) is known to participate in the control of cell proliferation, in the dephosphorylation of the retinoblastoma gene product (*pRb*), a tumor suppressor gene that plays an important role in cell-growth suppression and regulation of cell-cycle progression. Ceramide can act as an activator of transcription of the cyclooxygenase gene (Ballou *et al.*, 1992). Sphingosine (Faucher *et al.*, 1988) caused phosphorylation of the epidermal growth factor receptor on threonine 669 which was independent of protein kinase inhibition and this action of sphingosine might be associated with the conversion of sphingosine to ceramide (Goldkorn *et al.*, 1991). Furthermore, this phosphorylation is mediated by a membrane kinase (ceramide-activated protein kinase) which is activated by TNF-α in intact and in cell-free systems (Mathias *et al.*, 1991). Ceramide-activated protein kinase shares the substrate specificity of mitogen-activated protein kinase (MAP kinase) (Joseph *et al.*, 1993). Ceramide activates MAP kinase (Raines *et al*, 1993). Ceramide-activated protein phosphatase (CAPP) acts as a mediator of the action of ceramide. Ceramide-activated protein phosphatase (CAPP) serves as a serine/threonine protein phosphatase which is activated directly and specifically by ceramide but not by dihydroceramide (Dobrowsky

and Hannun, 1992) and is inhibited by okadaic acid (Dobrowsky *et al.*, 1993), a tumor promotor.

Sphingoid bases present in the colon may also help to protect against the development of colon cancer. Schmelz *et al* (1998) reported that both sphinganine and fumonisin B_1, a mycotoxin which blocks *de novo* sphingolipid biosynthesis and causes accumulation of sphinganine, induce apoptosis in HT-29 human colon cancer cells. Sphingosine inhibits protein kinase C (Hannun *et al.*, 1986a) which has been related with tumor promotion (Weinstein, 1988) and blocks induction of ornithine decarboxylase by phorbol esters (Gupta *et al.*, 1988, Enkvetchakul *et al.*, 1989). Sphingosine also inhibits the transformation of C3H10T1/2 cells (Borek *et al.*, 1991). Sphingosine and *N*-methylated derivatives inhibit growth of various human tumor cell lines *in vitro* (Stevens *et al.*, 1990a) and in nude mice (Endo *et al.*, 1991) and reduce the metastatic potential of a murine melanoma cell line (Okoshi *et al.*, 1991). In addition, Sweeney *et al* (1996) showed that sphingosine and its methylated derivative N,N-dimethyl sphingosine induce apotosis in a variety of human cancer cells including CMK-7, HL-60, U-937, HRT-18, MKN-74, and COLO-205. In contrast, primary cell cultures seem to be less susceptible to sphingosine than cancer cells. Sphingosine did not induce apoptosis in normal epithelial cells such as HUVECs or rat mesangial cells, but caused apoptosis in their transformed counterparts (Sweeney *et al.*, 1996). Thus, sphingolipids have the possibility to inhibit carcinogenesis through many mechanisms.

C. Knowledge of the types and concentrations of sphingolipids in foods is limited. Although evidence is now emerging which suggests that dietary

sphingolipids may protect against the development of disease, knowledge of the types and concentrations of sphingolipids in foods is limited. Sphingolipids are found in all eukaryotic and some prokaryotic cells (Merrill, 1991). However little is known about their concentrations in foods. Sphingomyelin (*N*-acylsphingosine-1-phosphocholine or ceramide phosphocholine) is a phospholipid located mainly in the outer leaflet of the plasma membrane of most mammalian cells (Parodi, 1997). The levels of sphingomyelin have been measured in the following foods: milk (100~200 nmol/ml) (Zeisel *et al.*, 1986), salmon (160 nmol/g), pork and beef tissues (350~390 nmol/g) and chicken (530 nmol/g) (Blank *et al.*, 1992). Kamath and Charles (1997) reported that the major phospholipid components of Swiss cheese whey lipid fractions were phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin.

Total lipids in milk are composed of 0.2 to 1.0% phospholipids. Sphingomyelin represents about 30% of total milk phospholipids and the concentration of sphingomyelin in milk was found to be about 100~200 nmol/mL (Zeisel *et al.*, 1986). However, the concentration of sphingomyelin in milk is affected by season and the cow's stage of lactation (Parodi, 1997). Lactic acid bacteria, particularly *Lactobacillus* and *Bifidobacteria*, are commonly used in the production of fermented milk. A study of the lipid composition of dairy starters reported that sphingomyelin is present in *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* (Chand *et al.*, 1992). Therefore, fermented milk products may be rich in sphingolipids. Furthermore, lactic acid bacteria such as *Lactobacilli* and certain components of fermented milk products have been shown to protect against the development of diseases (Malhorta, 1977, Goldin and Gorbach, 1980, International Agency for Research on Cancer, 1983, Bianchi Salvadori, 1986, Mutai and

Tanaka, 1987, & Ganjam *et al.*, 1997). Since sphingosine and ceramide can induce apoptosis, the protective effect of fermented milk products against diseases may be related, in part, to the dairy products' sphingolipid content.

Many studies reported that soy foods and certain components in soy products such as genistein protect against development of colon carcinogenesis (Akiyama *et al.*, 1987, Ogawara *et al.*, 1989, Teraoka *et al.*, 1989, Bourquin *et al.*, 1996, Thiagarajan *et al*, submitted). Ohnishi and Fujino (1982) reported that the amount of cerebroside in soybean was about 2 μmol/g. The protective effect of soy products against colon cancer may also be related to sphingolipid concentration.

II. OBJECTIVES

The specific objectives of this research are to:

A. Determine the concentrations of total sphingolipids and free sphingoid bases in soy fractions, nonfat dry milk, yogurt, and Swiss cheese.

B. Determine the effects of exogenous sphingolipids on growth and death of human colon cancer cells.

III. MATERIALS AND METHODS

Chemicals. Sphingolipids were purchased from Sigma (St. Louis, MO) and Matreya (Pleasant Gap, PA) and the other chemicals were obtained from Sigma (St. Louis, MO).

Foods. Yogurt (Low fat yogurt: custard style-thick & creamy, strawberry and vanila) was obtained from Yoplait Inc. (Minneapolis, MN). Swiss cheese was from Amish Country Cheese (Linwood, MI), nonfat dry milk and ultra-pasteurized coffee cream were from Kroger Co. (Cincinnati, OH). Full fat soy flakes, soy flour, and soy protein concentrate were from Central Soya (Fort Wayne, IN). Isolated soy protein with isoflavones was from Archer Daniels Midland Co. (Decatur, IL). The yogurt, Swiss cheese, and coffee cream were lyophilized in a freeze drier (Freeze Dry/Shell Freeze System, Labconco, Kansas City, Missouri). Nonfat dry milk and full fat soy flakes (whole soybeans) and the freeze-dried pellets from yogurt, Swiss cheese, and coffee cream were powdered using a mortar and pestle.

Colon cancer cell lines. The human colon adenocarcinoma cell line HT-29 and the human colon carcinoma cell line HCT-116 were purchased from American Type Culture Collection (Rockville, MD).

Determination of total lipids in foods. Total lipids in nonfat dry milk, yogurt, Swiss cheese, coffee cream, full fat soy flakes (whole soybeans), soy flour, isolated soy protein, and soy protein concentrate were extracted using chloroform/methanol (2:1, v/v). Chloroform/methanol (2:1, v/v) at 100 mL was added to

5g of dry materials and fat was solublized at 50 °C for 3 hours. Solvents and solubilized lipids were removed from substrates by Whatman filter paper and residues were dried at 60 °C for 24 h. The amounts of lipids were determined by gravimetric analysis.

Yogurt, Swiss cheese, and coffee cream which contain large amounts of moisture were freeze-dried to remove water. The freeze-dried materials of the foods were powdered using a mortar and pestle for determination of total lipids and sphingolipids. To measure moisture content in foods, raw food materials were dried at 60 °C for 24 h in VWR Scientific 1330 F Constant Temperature Oven (Forced air or gravity) (VWR Company, Philadelphia, PA) and the results were presented in Table 1.

Extraction of sphingolipids from foods for the determination of total sphingolipids. Sphingolipids were isolated by the one phase extraction method (Smith and Merrill 1995). The volume of 3 mL of chloroform/methanol (1:2, v/v) was added to the sample to be extracted. C_{20} sphingosine at 300 pmol as an internal standard was added to each sample to determine the concentrations of total sphingolipids. Samples were incubated at 37°C for 1 h in the shaker water bath (Shaker bath, Lab-Line Instrumentals, Inc., Melrose Park, IL) at 100g. After 1 h incubation, the sample was centrifuged at 800g for 10 min (Beckman Instruments, Inc., Fullerton, CA) and the clear supernatant was transferred to a new tube. This step was repeated for twice.

Three mL chloroform/methanol (2:1, v/v) was added to the pellet. After one hour incubation at 37°C in the shaker water bath, this extract was centrifuged and the clear supernatant was collected in the tube from the first step. This step was repeated for twice.

The pooled chloroform/methanol extracts from the above two steps were dried in a speed vacuum concentrator (AES 2000 Automatic Environmental Speed Vac® with Vapornet, Savant Instruments, Inc., Holbrook, NY). The dried extracts were dissolved in 1 mL of chloroform. After 1 h incubation at 37°C in the shaker water bath, the extracts were centrifuged at 800g for 10 min and clear supernatant was transferred to a new tube.

One mL of chloroform was added to the pellet. After 1 h incubation at 37°C in the shaker water bath, this pellet in chloroform was centrifuged and the clear supernatant was transferred to the supernatant collected in the tubes from the last step. The pooled chloroform extracts were dried in a speed vacuum concentrator.

Quantitation of total sphingolipids. After the one-phase extraction step, 2 mL of 0.5 N hydrochloric acid in methanol was added and the samples were incubated at 65°C in a dry bath (Barnstead/Thermolyne, Dubuque, IA) to hydrolyze the acyl groups. After 18 h incubation, samples were neutralized with 200 μL of 5 N ammonium hydroxide.

The samples were extracted with 1 mL of chloroform and 5 mL of water. This resulted in two phases of the samples: the top phase consisted of water and methanol and the lower phase consisted of lipids in chloroform. After the samples were centrifuged, the upper aqueous phase was removed (This step was repeated for twice). The chloroform layer was passed through a column filled with sodium sulfate to remove residual water. The chloroform was evaporated using a Speed Vacuum Concentrator for about 1 h. The samples were saponified by adding 1 mL of 0.1 M potassium hydroxide

in methanol/chloroform (4:1, v/v) and incubating the samples for 1 h in a shaker water bath at 37°C.

After the 1 h saponification, the samples were extracted with 1 mL of chloroform and 5 mL of water. This resulted in two phases of the samples: the top phase consisted of water and methanol and the lower phase consisted of lipids in chloroform. After the samples were centrifuged, the upper aqueous phase was removed (This step was repeated twice). The chloroform layer was passed through a column filled with sodium sulfate to remove residual water. The chloroform was evaporated using a speed vacuum concentrator for about 45 min. The samples were redissolved in 400 μL of mobile phase (methanol: 5 mM potassium phosphate, 87:13, v/v). The sphingoid bases in samples were derivatized with the addition of 200 μL of *o*-phthaldehyde in 3% borate buffer at pH 10.5. The samples were centrifuged at 10,000g for 10 min (Microcentrifuge, IEC/Micromax®) and the clear upper fraction was transferred to HPLC vials and the sphingoid bases were quantitated using High-Performance Liquid Chromatography (HPLC) (Merrill *et al.*, 1988, Riley *et al.*, 1994). The absolute amounts of total sphingolipids were determined by the reference to the internal standard, C_{20}-sphingosine.

The elution profiles of sphingosine, sphinganine, and C_{20}-sphingosine on C_{18} reverse-phase HPLC with the solvent of methanol: 5 mM potassium phosphate, pH 7.4 (87:13) were shown in Figure 4. Sphingosine, sphinganine, and C_{20}-sphingosine appeared at 12.5, 17.8, and 23.1 min respectively.

Quantitation of free sphingoid bases- sphingosine and sphinganine. Chloroform/methanol (1:2, v/v) at 1.9 mL was added to samples. C_{20}-sphingosine at 300

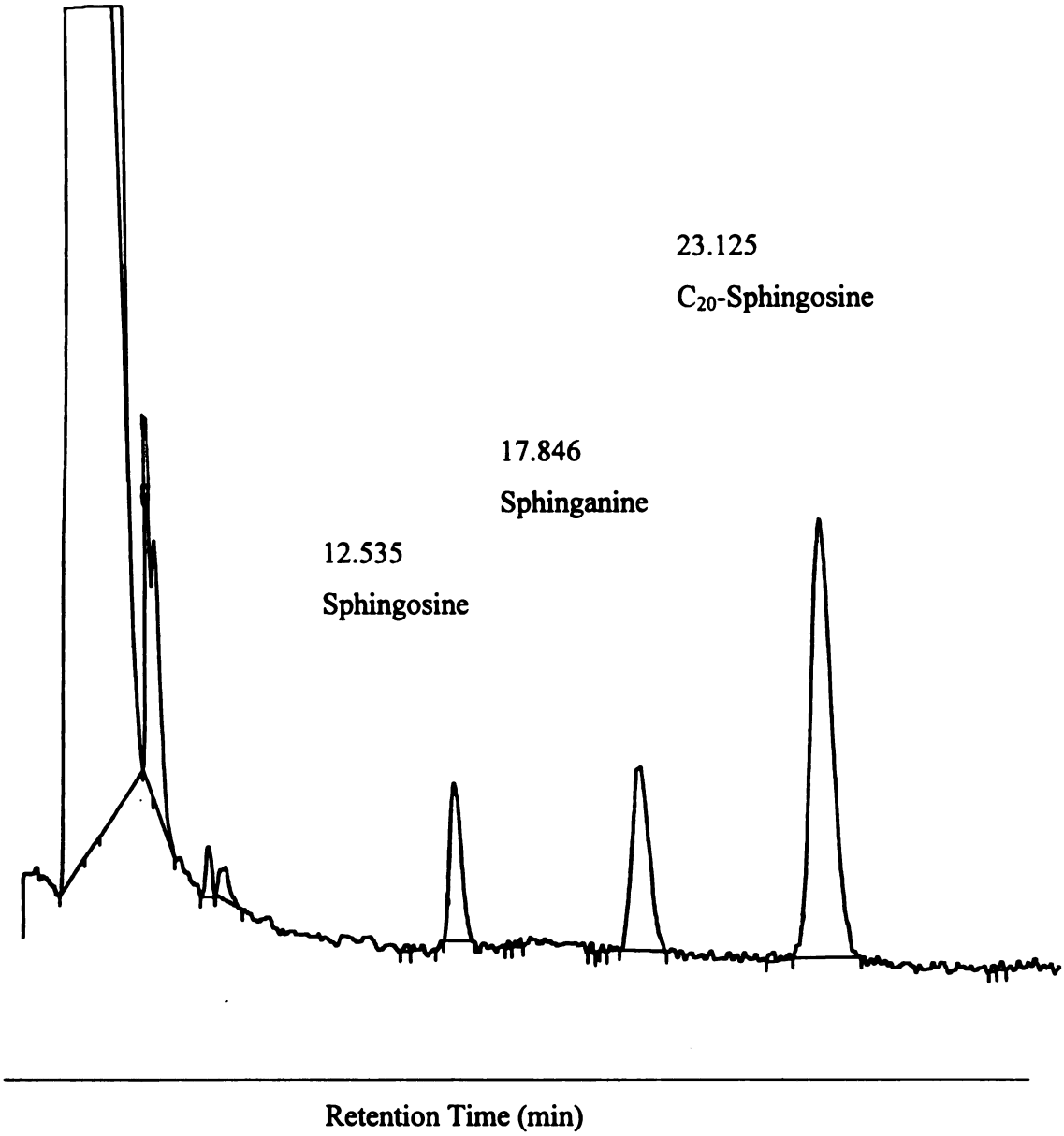

Figure 4. Elution profiles of sphingosine, sphinganine, and C_{20}-sphingosine on C_{18} reverse-phase HPLC.

pmol was added as an internal standard to samples and samples were mixed. After the addition of 100 μL of 2 N ammonium hydroxide, the samples were incubated at 37°C in a shaker water bath for 1 h. After 1 h saponification, the samples were extracted with 1 mL of chloroform and 5 mL of water. This resulted in two phases of the samples: the top phase consisted of water and methanol and the lower phase was a mixture of lipids in chloroform. After the samples were centrifuged at 800g, the upper aqueous phase was removed (This step was repeated for twice). The chloroform layer was passed through a column filled with sodium sulfate to remove residual water. The chloroform was evaporated using a speed vacuum concentrator for about 45 min. The samples were redissolved in 400 μL of mobile phase (methanol: 5 mM potassium phosphate, 87:13, v/v). The sphingoid bases in samples were derivatized with the addition of 200 μL of *o*-phthaldehyde in 3% borate buffer at pH 10.5. The samples were centrifuged at 10,000g for 10 min (Microcentrifuge, IEC/Micromax®) and the clear upper fraction was transferred to HPLC vials and the sphingoid bases were quantitated using High-Performance Liquid Chromatography (HPLC) (Merrill *et al.*, 1988, Riley *et al.*, 1994). The absolute amounts of free sphingoid bases were determined by the reference to the internal standard, C_{20}-sphingosine.

The elution profiles of sphingosine, sphinganine, and C_{20}-sphingosine on C_{18} reverse-phase HPLC with the solvent of methanol: 5 mM potassium phosphate, pH 7.4 (87:13) were shown in Figure 4. Sphingosine, sphinganine, and C_{20}-sphingosine were appeared at 12.5, 17.8, and 23.1 min respectively.

Culture of human colon cancer cells. Stock cultures of HT-29 and HCT-116 human colon cancer cells were grown in 100 mm culture dishes (Corning, Cambridge, MA) containing Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Dulbeco's Modified Eagle Medium was purchased from Gibco BRL (Life Technologies, Gaithersburg, MD).

Sphingolipid stock. Sphingosine and sphinganine were prepared as a complex with bovine serum albumin at concentrations of 10^{-4} M, $5x10^{-4}$ M, and 10^{-3} M. C_2-ceramide and C_2-dihydroceramide (cell permeable and short chain analog of naturally occurring ceramide and dihydroceramide) (Figure 5) were dissolved in ethanol as a stock solution at concentrations of 10^{-4} M, $5x10^{-4}$ M, 10^{-3} M, $2x10^{-3}$ M, and $5x10^{-3}$ M.

Total nucleic acid assay. To assess the effects of sphingolipids on cell growth and death, total nucleic acids were measured as previously described (Li *et al.*, 1990) and used as an index of cell number. Cells were seeded at a density of 3.0×10^5 cells/mL in 6-well dishes. HT-29 and HCT-116 cells were grown in 2 mL of Dulbeco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum for 24 or 36 h to insure that cells were in log phase before treatment with sphingolipids. After 24 or 36 h, the medium was replaced with DMEM supplemented with 1% fetal bovine serum and 20 μL of various concentrations of sphingolipids were added directly to the cell culture medium.

Subconfluent cells were incubated with sphingosine, sphinganine, C_2-ceramide, or C_2-dihydroceramide at final concentrations of 1, 5, 10, 20 and 50 μM for 0, 3, 12, 24, and 48 h. At each culture period of time, floating dead cells were removed by aspiration and the remaining live cells were rinsed with 1 mL of phosphate-buffered saline (PBS). Then

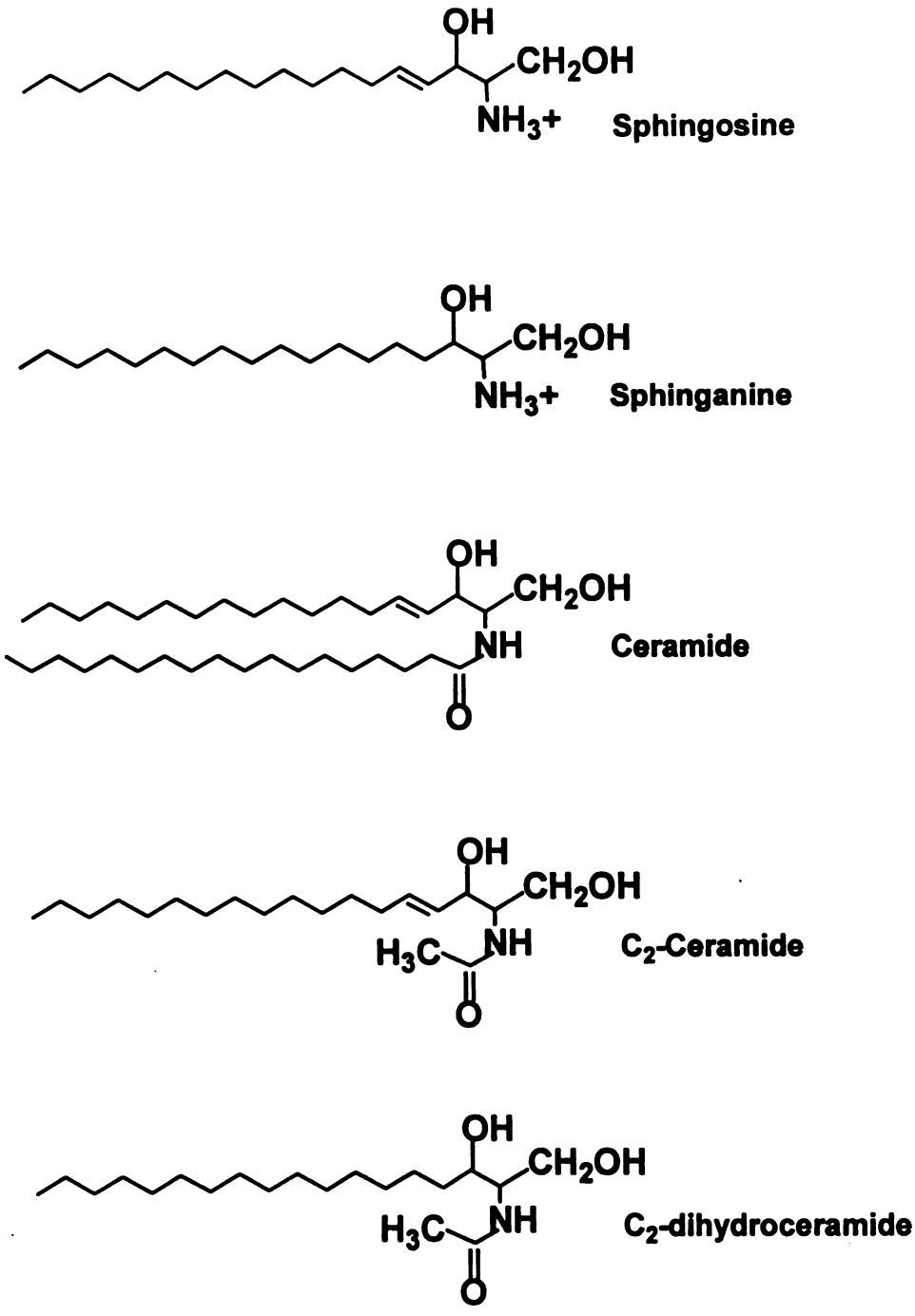

Figure 5. Structures of sphingoid bases and ceramides with or without the 4,5-trans double bond.

the cells were lysed with 1 mL of 0.1 M sodium hydroxide and the total nucleic acid concentration was determined by reading absorbance of the cell lysate at 260 nm using Gene Quant-RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ).

Examination of cellular morphology. Cells were treated with sphingolipids as described above and the cells were viewed and photographed using a Nikon inverted microscope (Nikon, Garden City, N.Y.) fitted with a Polaroid Micro Camera (Polaroid Corporation, Cambridege, MA).

Microscopic detection of apoptotic cells.. To determine whether sphingoid bases and ceramide kill cells via apoptosis or via a non-apoptotic pathway, cells were incubated with sphingoid bases and ceramide at 10, 20, and 50 μM for 0, 15, 30, 60, 120, or 180 min and 12, or 24 h. Cells were collected at each time and stained with acridine orange and ethidium bromide (Mishell *et al.*, 1980). Cells were photographed using a fluorescence microscope equipped with a camera under 10X plus 40X magnification with 400/490 nm excitation and 520 nm emission (Nikon Labophoto, Nikon Inc. Instrument Group, Garden City, N. Y.). Apoptotic and non-apoptotic cells were classified by the differences in their chromatin organization (Martin and Lenardo, 1998). Viable cells with normal nuclei appeared as containing bright green chromatin with organized structure. Viable cells with apoptotic nuclei appeared as containing bright green chromatin with highly condensed or fragmented structure. Nonviable cells with normal nuclei appeared as containing orange chromatin with organized structure. Nonviable cells with apoptotic nuclei appeared as containing orange chromatin with highly condensed or fragmented structure.

Statistical analysis. Data for cell growth as influenced by sphingolipids were analyzed by two-way factorial analysis of variance (ANOVA). After application of ANOVA to the data, the significance of differences in the means of total nucleic acid content in HT-29 and HCT-116 cells between control and treatment groups at specific culture periods were evaluated by multiple comparisons using the Bonferroni method. Differences were considered significant at $p < 0.05$.

IV. RESULTS

A. Sphingolipid concentrations in foods.

Determination of total lipids in foods. Total lipids in foods were expressed as percent of lipids on a dry weight basis (Table 1). Coffee cream, Swiss cheese, full fat soy flakes, and yogurt were relatively high in fat (54.5, 36.7, 23.1, 16.2 % respectively). Nonfat dry milk, soy flour, isolated soy protein, and soy protein concentrate contained 1.2, 2.7, 2.2, and 0.2 % fat, respectively on a dry weight basis.

Determination of sphingolipids in foods. The present study showed that most sphingolipids in foods are present as complex sphingolipids and little are present as free sphingoid bases (Table 2). The concentrations of total sphingolipids and free sphingoid bases (nmol/g of dry weight) were: nonfat dry milk (203 $\pm$ 75, 146 $\pm$ 78); yogurt (138 $\pm$ 55, 1.2 $\pm$ 0.3); Swiss cheese (167 $\pm$ 45, 6.5 $\pm$ 7.6); full fat soy flakes (609 $\pm$ 627, 2.6 $\pm$ 1.1); soy flour (610 $\pm$ 509, 1.6 $\pm$ 0.3); isolated soy protein (210 $\pm$ 112, 2.8 $\pm$ 1.7) (mean $\pm$ SD, n=4) (Table 2 and Figure 6). Sphingosine is the predominant free sphingoid base and long-chain base backbone of total sphingolipids in foods. The percentage of free sphingoid bases and sphingoid base backbones of food complex sphingolipids that are sphingosine and sphinganine is shown in Table 3. Sphingosine accounts for 90, 74, 91, 85, 98, and 96% of the sphingoid base backbone of total sphingolipids in nonfat dry milk, yogurt, Swiss cheese, full fat soy flakes, soy flour, and isolated soy protein, respectively (Table 3). Sphingosine accounts for 97, 69, 90, 64, 77, and 73% of free sphingoid bases in nonfat dry milk, yogurt, Swiss cheese, full fat flake, soy flour, and isolated soy protein, respectively (Table 3).

Table 1. Total lipids and moisture content in foods. Lipids are expressed on a dry weight basis. Data are mean ± SD (n=2).

Foods	% of lipids	Water (%)
Nonfat dry milk	1.2 ± 0.07	5.4 ± 0.3
Yogurt	16.2 ± 0.21	72.9 ± 0.002
Swiss cheese	36.7 ± 0.16	32.8 ± 6.7
Coffee cream	54.5 ± 1.35	72.2 ± 0.2
Full fat soy flakes	23.1 ± 0.14	3.4 ± 0.4
Soy flour	2.7 ± 0.14	4.5 ± 0.07
Isolated soy protein	2.2 ± 0.12	7.6 ± 0.02
Soy protein concentrate	0.2 ± 0.14	4.4 ± 0.04

Table 2. Sphingolipid concentrations in foods. Data are expressed on a dry weight basis and are mean ± SD (n=4).

Foods	Total Sphingolipids (nmol/g of dry weight)			Free sphingoid bases (nmol/g of dry weight)		
	Sphingosine backbone	Sphinganine backbone	Sum of sphingoid base backbone	Sphingosine	Sphinganine	Sum of sphingoid bases
Nonfat Dry Milk	179.2 ± 55.3	23.7 ± 27.1	202.9 ± 75.4	141.3 ± 76.1	4.9 ± 3.3	146.1 ± 77.8
Yogurt	104.4 ± 52.9	33.9 ± 11.9	138.3 ± 55.0	0.85 ± 0.3	0.4 ± 0.03	1.2 ± 0.3
Swiss Cheese	153.5 ± 46.5	13.4 ± 5.5	166.9 ± 45.1	6.2 ± 7.6	0.3 ± 0.04	6.5 ± 7.6
Full fat soy flake	558.6 ± 639.0	50.0 ± 41.1	608.7 ± 626.8	1.7 ± 1.1	0.9 ± 0.6	2.6 ± 1.1
Soy flour	600.7 ± 498.0	19.5 ± 13.2	610.4 ± 509.0	1.2 ± 0.2	0.7 ± 0.3	1.6 ± 0.3
Isolated soy protein	203.3 ± 110.2	6.5 ± 3.3	210.7 ± 112.0	2.1 ± 1.4	0.7 ± 0.3	2.8 ± 1.7

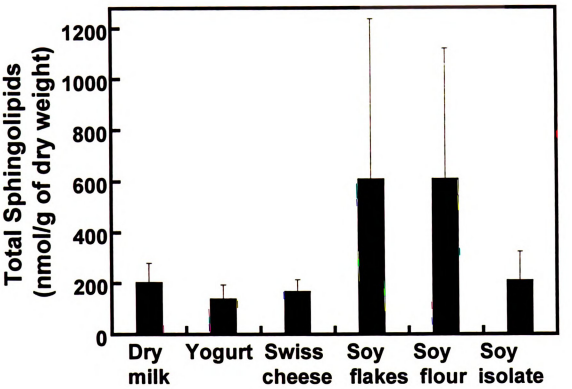

Figure 6. Total sphingolipid concentrations in foods. Data are espressed on a dry weight basis and are mean $\pm$ SD (n=4).

Table 3. Percentage of free sphingoid bases and sphingoid base backbones of food complex sphingolipids that are sphingosine and sphinganine.
Data are expressed on a dry weight basis and are mean ± SD (n=4).

Foods	Total Sphingolipids (%)		Free sphingoid bases (%)	
	Sphingosine backbone	Sphinganine backbone	Sphingosine	Sphinganine
Nonfat Dry Milk	89.8 ± 10.7	10.2 ± 10.7	96.7 ± 2.7	3.3 ± 2.7
Yogurt	74.0 ± 9.1	26.0 ± 9.1	69.4 ± 7.5	30.6 ± 7.5
Swiss Cheese	91.2 ± 4.8	8.8 ± 4.8	90.0 ± 7.7	10.4 ± 7.7
Full fat soy flake	84.5 ± 12.1	15.5 ± 12.1	64.4 ± 22.2	35.6 ± 22.2
Soy flour	98.4 ± 81.6	3.2 ± 2.2	76.7 ± 4.8	23.3 ± 4.8
Isolated soy protein	96.1 ± 2.0	3.9 ± 2.0	73.2 ± 8.0	26.8 ± 8.0

B. Differential sensitivity of human colon cancer cells to sphingoid bases and ceramide.

Sphingosine, sphinganine, and ceramide inhibit growth and cause death of HT-29 human colon cancer cells. Based upon the concentrations of total sphingolipids that were found in milk products in this study (~138-203 nmol/g) and assuming that the average American consumes ~730g of dairy products per day (USDA, 1994, Putnam and Allhouse, 1996), the sphingolipid concentration in the colonic lumen could reach >20 μM. Cells were incubated with sphingolipids at the concentrations where may be achieved after consumption of the foods based on this estimation.

To determine the effects of sphingolipids on growth and death of HT-29 human colon cancer cells, subconfluent cells were treated with sphingosine, sphinganine, C_2-ceramide, or C_2-dihydroceramide and the concentration of total nucleic acids was determined as an index of cell number. The concentration of total nucleic acids in control cultures doubled over 24 to 36 hours. Sphingosine, sphinganine, and ceramide caused concentration- and time-dependent decreases in total nucleic acids of HT-29 cells. Specifically, addition of sphingosine (Figure 7A) at 20 and 50 μM significantly reduced total nucleic acid concentrations within 24 hours by 50 and 66%, respectively ($p < 0.05$) compared to corresponding controls. Sphinganine (Figure 7B) at 10, 20 or 50 μM significantly reduced total nucleic acid concentrations within 24 hours by 55, 65, and 80%, respectively ($p < 0.05$) compared to corresponding controls. C_2-ceramide (Figure 7C) also caused a significant reduction in the total nucleic acid concentrations at 20 and 50 μM within 48 hours ($p < 0.05$). Incubation with C_2-ceramide at 50 μM for 48 h killed

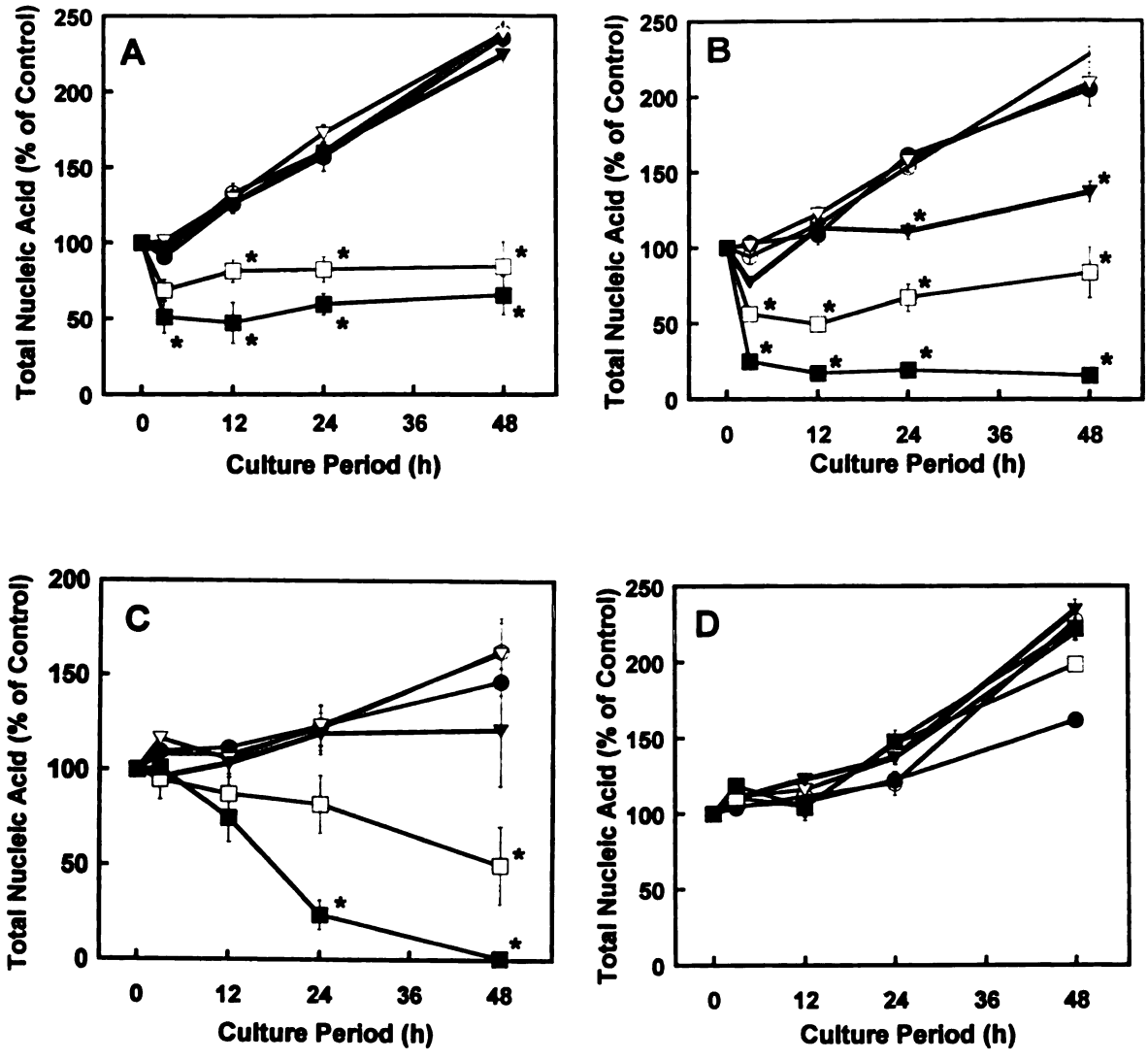

Figure 7. Sphingosine, sphinganine, and ceramide inhibit growth and cause death of HT-29 human colon cancer cells. Subconfluent cells were cultured with sphingosine (A), sphinganine (B), C_2-ceramide (C), and C_2-dihydroceramide (D) at 0 (●), 1 (○), 5 (▽), 10 (▼), 20 (❑), and 50 (■) μM for 3, 12, 24, or 48 h and total nucleic acid was determined as an index of cell number. Data are from two experiments and represent mean ± SEM (n=8). Means at each culture period with an asterik (*) are significantly different ($P < 0.05$) from the corresponding controls.

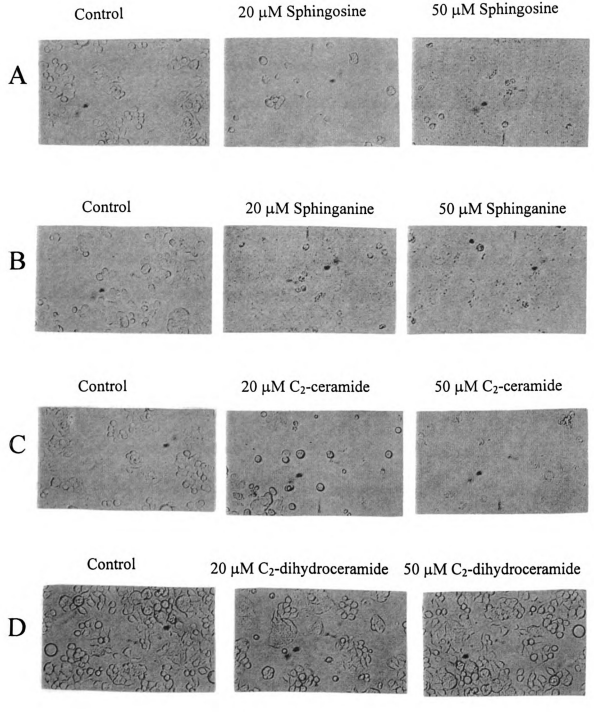

Figure 8. Sphingosine, sphinganine, and ceramide inhibit growth and cause death of HT-29 human colon cancer cells (photographs). Subconfluent cells were cultured with sphingosine (A), sphinganine (B), C_2-ceramide (C), and C_2-dihydroceramide (D) at 0, 20, and 50 μM for 24 h.

all cells. Unlike C_2-ceramide and the sphingoid bases, C_2-dihydroceramide did not reduce total nucleic acid concentrations (Figure 7D). Changes in cellular morphology caused by treatment of the cells with the sphingolipids for 24 h are shown in Figure 8.

Sphingosine, sphinganine, and ceramide inhibit growth and cause death of HCT-116 human colon cancer cells. Additional studies were conducted to examine the effects of sphingolipids on HCT-116 human colon cancer cells. The concentration of total nucleic acids in control cultures of HCT-116 cells doubled over 18 to 36 hours. Similar to the effects on HT-29 cells, sphingosine, sphinganine, and ceramide caused concentration- and time-dependent decreases in total nucleic acids of HCT-116 cells. Specifically, addition of sphingosine (Figure 9A) at 20 and 50 μM significantly reduced total nucleic acid concentrations within 24 h by 62 and 71%, respectively ($p < 0.05$) compared to corresponding controls. Sphinganine (Figure 9B) at 10, 20 or 50 μM significantly reduced total nucleic acid concentrations within 24 h by 55, 77, and 93%, respectively ($p < 0.05$) compared to corresponding controls. C_2-ceramide at 20 μM also caused a significant reduction in total nucleic acid concentrations within 24 hours by 33% and C_2-ceramide at 50 μM for 24 h killed all cells (Figure 9C). Unlike C_2-ceramide and the sphingoid bases, C_2-dihydroceramide did not reduce total nucleic acid concentrations (Figure 9D). Changes in cellular morphology caused by treatment of the cells with the sphingolipids for 24 h are shown in Figure 10.

Sphingoid bases and ceramide differentially affect death of human colon cancer cells. To determine whether sphingoid bases and ceramide kill cells via apoptosis or via a non-apoptotic pathway, cells were stained with acridine orange and

ethidium bromide and chromatin organization was examined under fluorescent light (Martin and Lenardo, 1998). Apoptotic cells were found in HT-29 and HCT-116 cells cultured with sphingosine at 20 μM and sphinganine at 10 and 20 μM for 12 and 24 h (Figure 11).

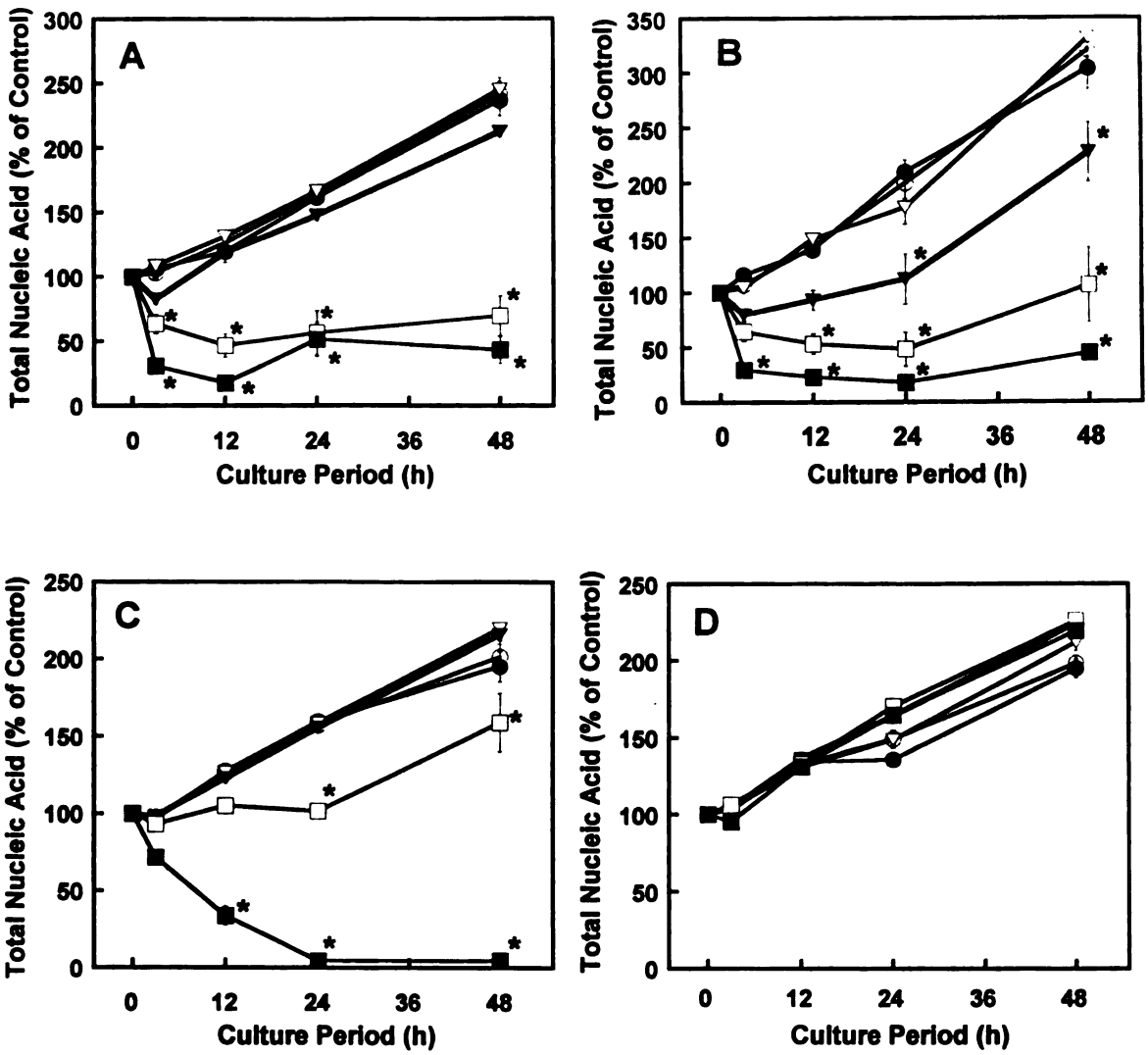

Figure 9. Sphingosine, sphinganine, and ceramide inhibit growth and cause death of HCT-116 human colon cancer cells. Subconfluent cells were cultured with sphingosine (A), sphinganine (B), C_2-ceramide (C), and C_2-dihydroceramide (D) at 0 (●), 1 (○), 5 (▽), 10 (▼), 20 (❑), and 50 (■) μM for 3, 12, 24, or 48 h and total nucleic acid was determined as an index of cell number. Data are from two experiments and represent mean ± SEM (n=8). Means at each culture period with an asterik (*) are significantly different ($P < 0.05$) from the corresponding controls.

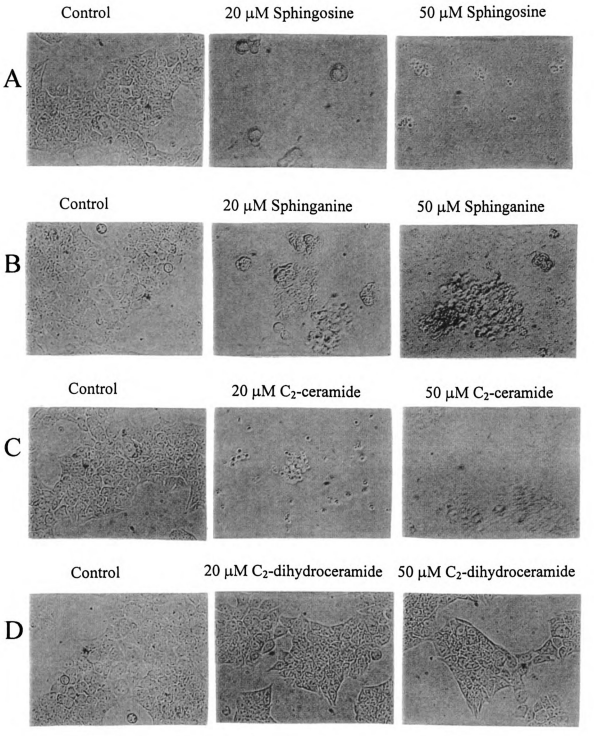

Figure 10. Sphingosine, sphinganine, and ceramide inhibit growth and cause death of HCT-116 human colon cancer cells (photographs). Subconfluent cells were cultured with sphingosine (A), sphinganine (B), C_2-ceramide (C), and C_2-dihydroceramide (D) at 0, 20, and 50 μM for 24 h.

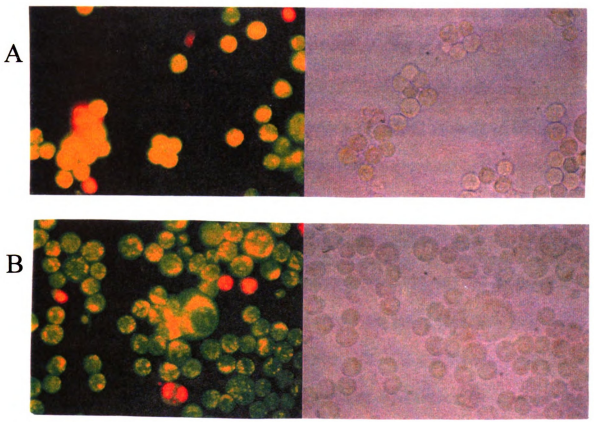

Figure 11. Sphinganine kill HT-29 cells by inducing apoptosis (photographs). Subconfluent cells were cultured either without sphinganine (A), or with sphinganine (B) at 10 μM for 12 h. Cells were stained with acridine orange and ethidium bromide. The right panels show cells under phase contrast and the left represents cells under florescent light.

V. DISCUSSION

Many studies suggest that specific foods and their components may protect against development of colon cancer. For example, soy foods (Akiyama *et al.*, 1987, Ogawara *et al.*, 1989, Teraoka *et al.*, 1989, Bourquin *et al.*, 1996, Hughes *et al.*, 1997, Thiagarajan *et al*, submitted) as well as milk and fermented milk products (Peters *et al.*, 1992, Kampman *et al.*, 1994) appear to protect against the development of colon carcinogenesis. Studies are underway to identify the component of these foods which provide the protective effects and their mechanisms of action. Recently dietary sphingolipids have gained attention for their potential to protect against the development of colon cancer (Dillehay *et al.*, 1994, Schmelz *et al.*, 1996, Schmelz *et al.*, 1997); however, little is known about the types and concentrations of sphingolipids in foods. In the present study sphingolipids were extracted from soy fractions, milk, and fermented milk products and quantitated via HPLC. The concentrations of total sphingolipids in full fat soy flakes, soy flour, and isolated soy protein were ~609, 610, and 210 nmol/g of dry weight, respectively. Although these concentrations are lower than those previously estimated for cerebroside (2 μmol/g) in soybeans (Ohnishi and Fujino, 1982), they indicate that sphingolipids are significant constituents of soy fractions. Furthermore, the data suggest that the processing steps to generate soy flour from full fat soy flakes using hexane extraction do not significantly influence the concentration of total sphingolipids. The present studies also show that the concentrations of total sphingolipids in nonfat dry milk, yogurt, and Swiss cheese are ~203, 138, and 167 nmol/g of dry weight,

respectively. Assuming that sphingomyelin is the major complex sphingolipid in milk, the concentration of total sphingolipids in nonfat dry milk (203 nmol/g) is comparable with the value reported for sphingomyelin in milk (100-200 nmol/mL) (Zeisel *et al.*, 1986). The concentrations of total sphingolipids in the fermented milk products are similar to that in nonfat dry milk suggesting that dairy starter cultures which contain sphingomyelin (Tamine and Deeth, 1980, Banwart, 1989, Chand *et al.*, 1992, Marshall, 1993) do not contribute significantly to the total sphingolipid concentration of the fermented foods. Sphingolipids are present in soy fractions and milk products primarily as complex sphingolipids; whereas, the simpler free sphingoid bases are present at low concentrations. Moreover, sphingosine is the predominant long-chain base accounting for ~64-97% of the free sphingoid bases and ~74-98% of the sphingoid base backbone of complex sphingolipids with sphinganine accounting for the balance.

Presumably dietary sphingolipids must reach the colonic tissue in order to inhibit the development of colon cancer. Schmelz *et al* (1994) reported that ~88% of dietary sphingomyelin is absorbed via the small intestine. Therefore, one route that dietary sphingolipids may reach the colon is via the blood. Alternatively, the remaining 12% of dietary sphingomyelin that is not absorbed passes directly into the colonic lumen (Schmelz *et al.*, 1994). This provides another more direct route by which dietary sphingolipids may reach the colonic cells. Based upon the concentrations of total sphingolipids that were found in milk products in this study (~138-203 nmol/g) and assuming that the average American consumes ~730g of dairy products per day (USDA,

1994, Putnam and Allhouse, 1996), the sphingolipid concentration in the colonic lumen could reach >20 μM.

The mechanism by which sphingolipids inhibit colon cancer is not clear. One possibility is that complex sphingolipids such as sphingomyelin are hydrolyzed to bioactive metabolites including sphingoid bases and/or ceramides. Assuming complete hydrolysis of complex sphingolipids which reach the colon, the concentration of sphingoid bases and/or ceramides that colonic cells may be exposed to via the lumen could reach >20 μM. This concentration of sphingoid bases and/or ceramides has been shown to inhibit growth and induce differentiation and/or apoptosis in a variety of tumor cells (Faucher *et al,* 1988, Okazaki *et al*, 1990, Stevens *et al*, 1990a, Stevens *et al*, 1990b, Michaelson, 1991, Goldkorn *et al*, 1991, Endo *et al*, 1991, Obeid *et al*, 1993, Sweeney *et al*, 1996).

The present study showed that sphingosine and ceramide at 20 and 50 μM and sphinganine at 10, 20, or 50 μM significantly inhibit growth and caused death of HT-29 and HCT-116 human colon cancer cells. In contrast, C_2-dihydroceramide which lacks the 4,5-trans double bond has no effects. These results suggest that the 4,5-trans double bond is necessary for inhibition of growth and induction of death by ceramide, but not by sphingoid bases (Figure 5). This finding is consistent with the results of previous studies which showed that short-chain ceramides caused apoptosis in many systems, while dihydroceramides which lack the 4,5-trans double bond were ineffective (Bielawska *et al.*, 1993, Obeid, *et al.*, 1993, Tepper *et al.*, 1995, Sawai *et al.*, 1995, Brugg *et al.*, 1996, Karasavvas *et al.*, 1996). In contrast, both sphingosine and sphinganine inhibit growth

and induce apoptosis in a variety of cell lines and tumor xenografts (Endo *et al.*, 1991, Ohta *et al.*, 1995, Jarvis *et al.*, 1996a, Sweeney *et al.*, 1996). Interestingly, previous studies have indicated that synthetic dihydrosphingomyelin (*N*-palmitoyldihydrosphingomyelin) (70%, $p < 0.0001$) was even more effective than synthetic sphingomyelin (*N*-palmitoylsphingomyelin) (52%, $p < 0.002$) at reducing the number of aberrant colonic foci in CF-1 mice treated with 1,2-dimethylhydrazine (Schmelz *et al.*, 1997). Taken together with our results, this suggests that synthetic dihydrosphingomyelin (and perhaps sphingomyelin) inhibits colon carcinogenesis via turnover in the colonic lumen to the free sphingoid base (sphinganine and/or sphingosine) which may in turn inhibit growth and cause death of colon cancer cells. Alternatively, Jarvis *et al* (1996a) showed that a combination of ceramide and sublethal concentrations of sphingosine or sphinganine was more effective at inducing DNA fragmentation in human myeloid leukemia cell lines HL-60 and U937 than ceramide alone. Therefore, complex sphingolipids may inhibit colon carcinogenesis via turnover to a mixture of sphingosine, sphinganine, and ceramide.

Incubation of cells with sphingosine at 20 μM and sphinganine at 10 and 20 μM for 12 h induced apoptosis; whereas, C_2-ceramide did not induce apoptosis. This finding raised a possibility of the utilization of sphingoid bases as a chemotherapeutic agent since many chemotherpeutic agents have been shown to kill susceptible cells by apoptosis (Kaufmann, 1989, Walker *et al.*, 1991, Shinomiya *et al.*, 1994, Havrilesky *et al.*, 1995, Huschtscha, *et al.*, 1996). Apoptosis is a form of programmed cell death which is either launched in response to specific stimuli such as cytokines, tumor necrosis factor α, and

the *fas* ligand (Nagata, 1992, Smith *et al.*, 1994), or activated in response to cell injury or stress (Gerschenson and Rotello, 1992, Michaelson, 1991). Cancer cells have been known to circumvent the normal apoptotic mechanisms to prevent their self-destruction due to their many mutations (Kerr *et al.*, 1994, Williams, 1991). Chemotherapeutic agents which induce apoptosis appear to have less side effects since the agents can selectively kill cancer cells with no cytotoxic effects on neighboring normal tissue (Kaufmann, 1989, Walker *et al.*, 1991, Shinomiya *et al.*, 1994, Havrilesky *et al.*, 1995, Huschtscha, *et al.*, 1996). The mechanisms by sphingoid bases induce apoptosis in human colon cancer cells has not well established. Wild-type p53 protein and *Bcl-2* oncogene were identified as two major endogenous regulators of apoptosis (Vogelstein and Kinzler, 1992, Oren, 1992, Lane, 1992, Reed, 1995, Korsmeyer *et al.*, 1995). Wild-type p53 protein induces cell death especially in response to DNA damaging events (Vogelstein and Kinzler, 1992, Oren, 1992, Lane, 1992). In contrast, *Bcl-2* oncogene show anti-apoptotic function (Reed, 1995, Korsmeyer *et al.*, 1995). Sphingosine might affect various systems to induce apoptosis. For example, sphingosine inhibits protein kinase C (Hannun *et al.*, 1986b), Na^+/K^+-ATPase (Oishi *et al.*, 1990), and phospatidic acid phosphohydrolase (Lavie and Liscovitch, 1990, Jamal *et al.*, 1991, Mullmann *et al.*, 1991). Sphingosine activates the epidermal growth factor receptor kinase (Faucher *et al.*, 1988, Wedegaertner and Gill, 1989) and other sphingosine-specific kinases (Pushkareva *et al.*, 1992). Sakakura *et al* (1996) showed that sphingosine induce apoptosis via down-regulation of *Bcl-2* oncogene in HL-60 cells. Thus, sphingoid bases might induce apoptosis by affecting multiple downstream targets.

Taken together, the results indicate that sphingolipids are significant constituents of dairy products and soy fractions and that the colonic concentrations of sphingoid bases and/or ceramide which may be achieved after consumption of soy fractions and fermented milk products are sufficient to inhibit growth and cause death of human colon cancer cells.

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