Sphingomyelin Consumption Suppresses Aberrant Colonic Crypt Foci and Increases the Proportion of Adenomas versus Adenocarcinomas in CF1 Mice Treated with 1,2-Dimethylhydrazine: Implications for Dietary Sphingolipids and Colon Carcinogenesis

Eva M. Schmelz, Dirck L. Dillehay, Sonji K. Webb, Alex Reiter, Jeanette Adams, and Alfred H. Merrill, Jr.

Departments of Biochemistry [E. M. S., A. H. M.], Pathology [D. L. D.], and Chemistry and the Emory Mass Spectrometry Center [A. R. J. A.], and Division of Animal Resources [D. L. D., S. K. W.], Emory University, Atlanta, Georgia 30322

ABSTRACT

Sphingolipids are hydrolyzed in the gastrointestinal tract to ceramide, sphingosine, and other metabolites that can modulate cell growth, differentiation, and apoptosis. To characterize the effects of dietary sphingolipids on colon carcinogenesis, female CF1 mice were administered 1,2-dimethylhydrazine and then fed an essentially sphingolipid-free diet supplemented with 0 to 0.1% (w/w) sphingomyelin (SM) purified from milk. As was found in a previous pilot study (D. L. Dillehay et al., J. Nutr., 124: 615—620, 1994), SM (0.1%) reduced the number of aberrant colonic crypt foci (by 70%, P < 0.001) and aberrant crypts per focus (by 30%, P < 0.003), which are early indicators of colon carcinogenesis. In longer term studies, SM had no effect on colon tumor incidence or multiplicity; however, up to 31% of the tumors of mice fed SM were adenomas, whereas all of the tumors of mice fed the diet without SM were adenocarcinomas. These findings demonstrate that milk SM suppresses the appearance of more advanced, malignant tumors as well as early markers of colon carcinogenesis. Although the sphingolipid content of foods has not been widely studied, several foods (e.g., milk and soybeans) contain the sphingolipid levels used in these investigations; therefore, this class of compounds could be significant contributors to the cancer preventive effects of some foods.

INTRODUCTION

Colon cancer is the second leading cause of cancer mortality in the United States. Genetic predisposition and environmental factors, such as diet, are thought to affect colon carcinogenesis, and a large number of studies have explored the relationships between specific nutrients and colon cancer (reviewed in Refs. 1–6). However, relatively few of the thousands of compounds in food have been evaluated for their effects on carcinogenesis, which may account for the current confusion regarding the relationships between diet and cancer.

Sphingolipids are prominent among the components of food that warrant investigation because they modulate processes that are important in carcinogenesis: cell growth, differentiation, and apoptosis (7, 8). The sphingolipid backbones (sphingosine, sphingosine 1-phosphate, sphingosylphosphorylcholine, ceramide, and ceramide 1-phosphate) are highly bioactive compounds that have been shown in studies with cells in culture to stimulate (9) or inhibit (10, 11) cell proliferation, enhance (12) or inhibit (13) cell differentiation, and induce cell death (10), in some cases via apoptosis (14). Sphingolipids have served as messengers in the signaling pathways for growth factors, cytokines, neurotrophic factors, and other agents (15).

In addition to these intracellular signaling pathways, cells of all regions of the gastrointestinal tract are exposed to ceramides, sphingosine, and other bioactive metabolites during the digestion of dietary sphingolipids (16). To determine if dietary sphingolipids affect intestinal cells, and especially, if they alter the response of colonic cells to carcinogens, SM3 was purified from powdered milk4 and fed to female CF1 mice that had been treated with DMH to induce colon tumors (17). SM reduced the number of aberrant colonic foci (an early marker of colon carcinogenesis) and decreased the number of animals with tumors; however, the reduction in tumor incidence was only marginally significant (P = 0.08), perhaps because too little SM was available for a thorough feeding study.

In the follow-up investigations described in this report, we have used a preparation of SM that is available commercially (purified from buttermilk) to establish more conclusively that feeding SM to DMH-treated CF1 mice reduces the number of aberrant colonic crypt foci and the number of crypts per focus. The consumption of SM was also discovered to shift the types of tumors induced by DMH from exclusively adenocarcinomas to a combination of adenomas and adenocarcinomas. These findings provide further evidence that this major class of dietary lipids could play a role in the associations between diet and cancer.

MATERIALS AND METHODS

Animals. Female CF1 mice (5 weeks of age and virus antibody free) were obtained from Charles Rivers Laboratories (Portage, MI) and kept at 23°C ± 2°C with 50—60% relative humidity and a 12-h light/dark cycle. Mice were housed in microisolator cages (five per cage) with hardwood chip bedding and had free access to water and, for the first week (for acclimatization), were fed Purina Rodent Chow 5001 (Ralston Purina, St. Louis, MO) ad libitum. The animals were weighed weekly and monitored closely for signs of disease. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee and conducted according to National Research Council guidelines (18).

Sphingomyelin. SM was isolated from powdered, low-fat milk as described previously (16) and from buttermilk (uncultured); the latter was purchased from Matreya, Inc. (Pleasant Gap, PA). The two SM preparations were compared by thin-layer chromatography and high-performance liquid chromatography (16) and by mass spectrometry using a JEOL JMS-SX102 SX102/A/E, five-sector, tandem (MS1-MS2-MS3) mass spectrometer (19, 20). Full-scan negative ion FAB mass spectra were acquired using MS1 and frit-FAB in which the solvent was 2:1 CHCl3/methanol containing 1% triethanolamine. Precursor ions were produced by bombarding the samples with 6-keV xenon atoms at a gun emission current of 5 mA, which were then accelerated to 10 keV. Tandem mass spectrometric experiments (MS-MS

The abbreviations used are: SM, sphingomyelin; DMH, 1,2-dimethylhydrazine; FAB, fast atom bombardment; MS, mass spectrometry.

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2 To whom requests for reprints should be addressed, at Department of Biochemistry, 4113 Rollins Research Center, Emory University, Atlanta, GA 30322. Phone: (404) 727-5978; Fax: (404) 727-3954.
3 The abbreviations used are: SM, sphingomyelin; DMH, 1,2-dimethylhydrazine; FAB, fast atom bombardment; MS, mass spectrometry.
4 Sphingolipids can be isolated from powdered milk because they are mostly part of the membranes rather than the milkfat droplet, and a large portion is retained in "lowfat" milk and milk products.

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Experiments were performed by mass- and energy-selecting the precursor ions using MS1 and then colliding the ions with helium in a collision cell located between MS1 and MS2. The MS-MS spectra were acquired by linking MS2 and MS3 together in a scan in which the magnets and electrostatic analyzers were held at a constant ratio of magnetic field strength to electrical field strength (B:E).

Experimental Diets and Carcinogen Treatment. The mice were randomly divided into the experimental groups and fed a semipurified AIN 76A diet (Ralston Purina) after 7 days of acclimatization. The sphingolipid content of this diet is <0.005% (w/w; Ref. 17) and serves as an essentially sphingolipid-free control. 1,2-Dimethylhydrazine·HCl (DMH) was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted before each injection in 1 mm EDTA. Fifty mice/group (in the carcinogenesis study; 15/group in the aberrant crypt study) were injected i.p. with 0.5 ml of DMH (20 mg of DMH/kg body weight in the carcinogenesis study; 40 mg/kg for the aberrant crypt study) once a week for 6 weeks. These dosages of DMH are comparable to previous studies and were selected to target a tumor incidence of approximately 50% (21, 22).

One week after the final injection of DMH, the diets were supplemented with 0, 0.025, 0.05, and 0.1% SM (by weight). The SM was ground into a fine lipid-free control. 1,2-Dimethylhydrazine·HCl (DMH) was purchased from Sigma Chemical Co. (St. Louis, MO) and diluted before each injection in 1 mm EDTA. Fifty mice/group (in the carcinogenesis study; 15/group in the aberrant crypt study) were injected i.p. with 0.5 ml of DMH (20 mg of DMH/kg body weight in the carcinogenesis study; 40 mg/kg for the aberrant crypt study) once a week for 6 weeks. These dosages of DMH are comparable to previous studies and were selected to target a tumor incidence of approximately 50% (21, 22).

One week after the final injection of DMH, the diets were supplemented with 0, 0.025, 0.05, and 0.1% SM (by weight). The SM was ground into a fine powder and thoroughly mixed into the AIN 76A diet at the stated percentages, which were confirmed by high-performance liquid chromatography (23). The diets were prepared monthly and stored at 4°C (SM is stable under these conditions).

Analyses of Aberrant Colonic Crypts. After 4 weeks of feeding the experimental diets (5 weeks after the last injection of DMH), the mice were killed by CO2 asphyxiation, and the large intestines were removed, flushed with cold Tris [0.05 M Tris (hydroxymethyl) amino methane-HCl, pH 7.6, at 4°C], opened longitudinally, fixed overnight in 10% neutral buffered formalin, and stained with 0.2% methylene blue for 20 to 40 min. Aberrant crypt foci and the number of crypts per focus were measured by light microscopy at 40- or 100-fold magnification (24).

Carcinogenesis Study. After 34 weeks of feeding, blood was found in almost all of the feces sampled; therefore, all the animals were killed by CO2 asphyxiation. The large intestines were removed, flushed with cold Tris (0.05 M, pH 7.6 at 4°C), and opened longitudinally; the tumor number, location, and size were noted. The tumors were evaluated histologically after being fixed in formalin, embedded in paraffin, sectioned at 5 μm, and stained with H&E. In a blinded fashion, the tumors were classified as adenomas or adenocarcinomas, as described by Elsayed and Shamsuddin (25). Serum was collected from the 0 and 0.1% SM groups and analyzed for standard clinical chemistry parameters by a commercial lab.

Statistical Analyses. Statistical analyses were conducted using the Instat software (version 1.0; Instat, San Diego, CA). Differences in the weight and number of aberrant foci and tumors were evaluated by Student's t test after ANOVA analysis. The data on tumor composition comparisons were evaluated by Fisher’s exact test. Regression analyses were used for the calculation of correlations. Differences were considered significant at P ≤ 0.05.

RESULTS

Characterization of the SM Used in These Studies. As part of the follow-up to our previous study (17), the molecular subspecies were analyzed for the SM isolated by our laboratory from powdered milk (16) and a preparation from buttermilk (uncultured) that is available commercially (Matreya, Inc.). The mass spectrometric analyses of these samples are shown in Fig. 1, A and B. All of the molecular species from both samples were also collisionally activated in MS-MS experiments to confirm that they were SMs and to identify the components of isomers that produce the same m/z.

One example of a MS-MS spectrum of the precursor ion of m/z 616 from the full-scan FAB spectrum of the buttermilk sample is shown in Fig. 1C. This MS-MS spectrum was easily interpreted to be the spectrum of the (M-86)− ion of N-palmitoyl-(4E)-sphingosyl phosphorylcholine (d18:1/16:0; see structure in Fig. 1D) plus small amounts of the M-60 fragment ion for the d18:1/14:1 homologue (19). As has been reported previously (26), milk SM is composed of mostly sphingosine (d18:1/14:0) with saturated fatty acids (16:0, m/z 616; 22:0, m/z 700; 23:0, m/z 714; and 24:0, m/z 728). Within each cluster there are also small amounts of SM with sphinganine (d18:0) rather than sphingosine (d18:1). The higher m/z ions are mainly from the M-60 and M-15 fragmentations of...
This same series (i.e., m/z 642, 726, 740, and 754 for M-60, and m/z 687, 771, 785, and 799 for M-15). Thus, the MS-MS analyses provided unequivocal structure assignments and confirmed that both samples were composed of identical molecular species.

The SM preparations were also pure by thin-layer chromatography and analyses of the sphingoid base backbones by high-performance liquid chromatography (data not shown), which confirmed that sphingosines (d18:1; rather than sphinganines, d18:0) were the major sphingoid base backbones.

**General Characteristics of the Mice Fed SM.** The mice fed varying amounts of SM (from buttermilk) for a long term exhibited no differences in weight gain (Fig. 2). The pause in growth during the 10th week was due to a problem in the handling of the water bottles, but the animals quickly returned to their normal weight without any signs of illness after this was corrected. At the end of the study, the body weights were 53.2 ± 1.3, 51.1 ± 1.0, 51.6 ± 1.1, and 53.1 ± 1.6 g (mean ± SE) for the groups supplemented with 0, 0.025%, 0.05%, and 0.1% SM, respectively. Analyses of blood collected from mice in the 0 and 0.1% SM groups at the end of the study did not reveal differences in blood urea nitrogen, creatinine, glucose, albumin, globulin, or electrolytes (data not shown). Thus, as in our previous study (17), SM feeding did not appear to have deleterious effects on the animals.

**Effects of Dietary SM on the Appearance of Aberrant Colonic Crypts.** At 0.1% of the diet, buttermilk and powdered milk SM reduced the appearance of aberrant colonic crypt foci by 66 and 70%, respectively (P < 0.001; Fig. 3). There were approximately 30% fewer aberrant crypts per colonic focus in both SM groups (P = 0.0035 for milk powder SM and P = 0.0003 for buttermilk SM).

Most of the aberrant crypts were localized in the distal part of the colon, some were in the middle, and only a few were detected in the anterior portion (and these were seen only in the 0% SM group). There was no correlation between the weight of mice and the number of aberrant crypts (data not shown).

**Effect of SM on DMH-induced Colon Carcinogenesis.** All groups had the same tumor incidence and multiplicity (24.5, 19.6, 25.0, and 25.5%, and 1.3, 1.3, 1.3 and 1.2 tumors per tumor-bearing animal for 0, 0.025, 0.05, and 0.1% SM, respectively). The colonic tumors were all found in the middle and distal portions of the colon and ranged in size from 1–5 mm in diameter. Macroscopically, the tumors were sessile or pedunculated; microscopically, 57 colonic tumors were observed: 10 benign polypoid adenomas, as indicated by the presence of an intact muscularis mucosa (Fig. 4A); and 47 adenocarcinomas (usually large), which were characterized by invasion of neoplastic cells through the muscularis mucosa (Fig. 4B).

The difference between the mice fed SM-containing diets versus the control surfaced when the distribution of tumor types was compared (Fig. 5). All of the tumors of the mice fed the control diet were adenocarcinomas, whereas every group fed SM had a combination of adenomas and adenocarcinomas. This shift in the proportion of adenomas versus adenocarcinomas was significant (P = 0.043) for the 0.05% SM group and marginally significant (P = 0.075) for the 0.025% SM group.

**DISCUSSION**

The results of these studies support the hypothesis that dietary sphingolipids suppress colon carcinogenesis, perhaps through their hydrolysis to the bioactive backbones. The ability of sphingoid bases to affect transformation was initially proposed by Hannun et al. (27), based on the discovery that sphingosine is a potent inhibitor of protein kinase C. Borek et al. (28, 29) next found that sphingosine and sphinganine inhibit the induction of transformed foci in C3H 10T½ cells exposed to γ-irradiation and phorbol esters, and many types of cells in culture are now known to undergo change in growth, differentiation, and apoptosis upon exposure to various sphingolipids (30). Studies in vivo (31, 32), topical application of sphingosine to mouse skin inhibited the induction of ornithine decarboxylase by phorbol esters, a marker of tumor promotion. Subsequent experiments did not find that sphingosine blocked the development of skin tumors (33), but it is possible that the high amounts of sphingosine and ceramide that are already in skin (34) complicate the use of the skin tumor model. The administration of a number of sphingolipids i.p. or i.v. have been shown to block the growth and metastasis of
implanted tumors (35–37). As far as we are aware, our pilot experiments (17) constitute the only previous investigation of the effects of dietary sphingolipids on cancer.

The current investigation was possible because SM (from butter milk) has been recently made available commercially in the large quantities (>100 g) that are needed for long-term feeding studies. This preparation is structurally identical to the SM that we isolated from powdered milk and caused the same percentage of reduction in aberrant colonic crypts at 0.1% SM (66%) as the preparation from powdered milk (70%), which also agrees well with the 50% reduction by 0.05% SM in the previous feeding experiment (17). Furthermore, the SM preparations are highly pure (based on MS-MS and other analyses); therefore, their effects are likely to be due to SM per se and not a contaminant of the preparations.

Aberrant colonic crypt foci are one of the earliest morphological changes of the rodent colonic cells to dysplasia. It is thought that a percentage of these lesions will eventually develop into adenomas and adenocarcinomas (38–40), which makes them useful biomarkers for studies of agents that may inhibit (or enhance) colon carcinogenesis. Nonetheless, caution must be exercised in extrapolating findings with this biomarker because several agents that have affected the number of aberrant crypt foci have not had the same effect on development of tumors (41, 42). It has been suggested that the number of aberrant crypts per focus correlates better with the subsequent development of tumors (42). Our studies with SM have found significant reductions in both the number of aberrant colonic crypt foci and the number of aberrant crypts per focus.

Unlike our pilot study, which indicated that SM decreased the number of tumors induced by DMH, this investigation found that SM increased the portion of tumors that are histologically characterized as

**Fig. 4. Photomicrograph of the types of colonic tumors.** A, colonic mucosa containing a polypoid adenoma with an intact muscularis mucosa (M) at ×100; B, an adenocarcinoma with invasion (arrow) through the muscularis mucosa (M) at 100-fold magnification.

**Fig. 5. Number of tumors classified histologically as adenomas or adenocarcinomas in C57BL/6J mice treated with DMH and fed SM in the diet at percentages (w/w) shown.** The statistical significance of the differences between the designated groups relative to the 0% SM control were: *, P < 0.05; and **, P < 0.05.
INHIBITION OF COLON CARCINOGENESIS BY SPHINGOMYELIN

Intestinal Lumen

Colonic cell

<table>
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<td>Growth stimulation.</td>
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**Fig. 6.** Sphingolipid digestion in the lumen of the intestinal tract, uptake of metabolites, cellular metabolism, and possible effects on colonic cells. As described in the text, each of the events illustrated in this diagram have been noted by various studies; however, their integration to explain the observations in this manuscript is still hypothetical.

adenomas rather than the more malignant adenocarcinomas (Fig. 5). Colorectal tumors are thought to begin as adenomas and progress to adenocarcinomas through further genetic mutations (such as mutations in DCC and p53 tumor suppressor genes; Ref. 43); therefore, SM feeding might prevent the progression of adenomas to adenocarcinomas, although we cannot exclude the possibility that it is capable of promoting reversion of adenocarcinomas to adenomas. Since the shift in tumor type was statistically significant (P < 0.05) in the 0.05% SM group but not for the mice fed 0.1% SM, it is possible that high levels of SM are less effective; however, a more extensive dose-response study should be conducted before drawing this conclusion.5

Although there have been few analyses of the levels of sphingolipids in food, milk contains almost 0.1 mg of SM per ml (0.5 to 1 mg/g of dry milk) (44), pork and beef contain approximately 0.25 mg/g, and chicken has nearly 0.4 mg/g (45). Thus, these foods contain 0.025 to 0.1% SM, which compares well with the level (0.05% SM) that suppressed both the aberrant crypt foci and the development of adenocarcinomas. It warrants mention that the basal AIN 76A diet is starch, and others) being essentially sphingolipid free (17). Although there have been few analyses of the levels of sphingolipids in food, milk contains almost 0.1 mg of SM per ml (0.5 to 1 mg/g of dry milk) (44), pork and beef contain approximately 0.25 mg/g, and chicken has nearly 0.4 mg/g (45). Thus, these foods contain 0.025 to 0.1% SM, which compares well with the level (0.05% SM) that suppressed both the aberrant crypt foci and the development of adenocarcinomas. It warrants mention that the basal AIN 76A diet is starch, and others) being essentially sphingolipid free (17).

Why did the first experiment indicate that SM decreased the tumor number but the second showed a shift in tumor type? As already emphasized, these results are probably not due to differences in the SM preparations; however, the two experiments differed in another way. In the first study, we were unable to isolate enough SM for the entire time course; therefore, SM was added to the diet for only the first 21 weeks. It is possible that some SM metabolites (such as ceramide) inhibit the development of adenocarcinomas, but others (such as lyso-SM, which can be produced by bacterial enzymes and is mitogenic; Refs. 46 and 47) stimulate the growth of adenomas. In support of this possibility, SM feeding increased proliferation of colonic cells in the initial study (17) but only for DMH-treated mice. Fig. 6 summarizes current information about sphingolipid metabolism and cell regulation that may be relevant to the findings of this study. Some SM escapes digestion in the upper intestinal tract and is hydrolyzed in the colon (perhaps, involving intestinal microflora) to the lipid backbones ceramide and sphingosine (and possibly sphingosylphosphorylcholine, lyso-SM), and these are taken up by colonic cells (16). These sphingolipids and their metabolites are known to affect a wide spectrum of cellular targets and cell behaviors, from the regulation of growth and differentiation to apoptosis. The immediate targets of these sphingolipids are still being elucidated (recently reviewed in Refs. 8 and 15), but they have been suggested to include growth inhibition due to the inhibition of protein kinase C (10, 27) and induction of p53 dephosphorylation by sphingosine (48) or ceramide (14); inhibition of growth and induction of apoptosis by activation of phosphoprotein phosphatase(s) (49) and kinases (50) by ceramide; and increases in intracellular calcium (and growth stimulation) by sphingosine, sphingosine 1-phosphate, and sphingosylphosphorylcholine (9, 47, 51). Therefore, the simplest explanation for the findings of our two studies is that the sphingosine and/or ceramide derived from dietary SM suppress the development of aberrant colonic crypts and adenocarcinomas by inhibiting growth or inducing differentiation and/or apoptosis. When other mitogenic sphingolipid metabolites (such as lyso-SM) are produced, they may diminish these suppressive effects. Unfortunately, it is not possible to test these hypotheses by feeding the SM metabolites (lyso-SM, ceramide, or sphingosine) because these compounds are more rapidly taken up by the upper gastrointestinal tract and do not seem to reach the colon (16).

Dudeja et al. (52) have noted that one of the first changes in rat colonic cells induced by DMH is a decrease in membrane fluidity that appears to be due to increases in membrane SM. The elevation in SM was associated with an increase in SM synthesis and a reduction in the activity of the (neutral) sphingomyelinase. Thus, it is possible that some forms of neoplasia involve defect(s) in the sphingolipid signaling pathway(s) that regulate growth arrest and apoptosis; if so, an exogenous source of sphingolipid (i.e., via the diet) might be able to bypass the defect. Wright et al. (53) have recently described a cell line that is resistant to tumor necrosis factor α- and UV irradiation-induced cell death due to a defect in the activation of sphingomyelinase and the 24-kDa apoptotic protease, and the addition of exogenous ceramide bypassed the defect to induce apoptosis in the cells. Therefore, the effects of dietary SM in our studies may involve a similar bypass of aberrant sphingolipid signaling. If this is the case, this mechanism could be highly relevant both to understanding the relationships between diet and cancer and to the development of new approaches to cancer chemotherapy.

REFERENCES


5 These dose-response relationships might have been more clear if we had used a protocol that increased the number of animals that developed tumors (for example, by using higher amounts of DMH); however, because so little is known about the effects of dietary sphingolipids, we were attempting to induce tumors in about one-half of the mice in the control group so that increases as well as decreases in tumor incidence could be detected.

6 E. M. Schmeltz and A. H. Merrill, Jr., unpublished observations.

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INHIBITION OF COLON CARCINOGENESIS BY SPHINGOMYELIN


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