SPHINGOLIPIDS IN THE CHEMOPREVENTION OF COLON CANCER

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1. ABSTRACT

Sphingolipids were first described more than 100 years ago by the physician Thudicum who named the fatty substance he found in brain 'sphingosin'. Among multiple other functions, sphingolipids are lipid messengers in the signaling pathways of growth factors, cytokines, cellular stresses and others. As such, they are involved in the regulation of a wide spectrum of processes that modulate cell growth and cell death. These functions may be beneficial in cancer cells that escape growth regulation and exhibit unlimited proliferation. The effects of exogenous sphingolipids on cancer cells in vitro have been well documented; however, the effects of sphingolipids in vivo are less well understood. Since the mechanisms sphingolipids utilize in the prevention of cancer may be different from those in cancer treatment-modaluption of cell growth versus induction of cell death- this review will focus on the known effects of orally administered sphingolipids in the prevention of colon cancer in different rodent models, and discuss the effect of sphingolipid metabolites on changes in cell proliferation and cell death that are important events in early carcinogenesis.

2. INTRODUCTION

2.1. Structure of sphingolipids

Sphingolipids can be found in all eukaryotic cells. They are primarily localized in cellular membranes, but are also found in lipoproteins, milk fat globule membranes and other functional structures such as the lamellar permeability barrier in skin. Sphingolipids are composed from a sphingoid base, an amid-bound fatty acid, and a headgroup. As shown in Figure 1, the most common sphingoid base in complex sphingolipids of mammalian origin is sphingosine, although smaller amounts of sphinganine can also be found. More structural variations in the backbone can be found in plants, such as additional double bonds (4, 8-sphingadiene) or double bonds on C9-C19. The amid-bound fatty acids also vary in their carbon chain length (C14-C24), saturation grade (in mammalian cells mostly saturated), and α-hydroxylation. Addition of simple to rather complex headgroups forms more complex sphingolipids. In Figure 1, the structures of headgroups of sphingolipids used in our studies are shown. Altogether, variations in these components make sphingolipids the structural most diverse class of membrane lipids.

2.2. Sphingolipids as lipid second messengers

About 15 years ago, a new concept of sphingolipids as second messengers, mediating the response of cells to exogenous regulatory compounds was introduced with the description of the sphingomyelin cycle (1). Extracellular agents such as growth factors, cytokines (PDGF, TNF-α, IL-1β) activate a sphingomyelinase, an enzyme in the sphingolipid degradation pathway (Figure 2A). This has also been demonstrated in response to stresses such as UV light, γ-irradiation, serum deprivation, hypoxia and heat, and chemotherapeutic agents (daunorubicin, vincristine) (see recent reviews 2-3). Growth factors such as PDGF not only activate sphingomyelinase but also a ceramidase to generate sphingosine, and sphingosine kinase to phosphorylate sphingosine to sphingosine-1-phosphate. More recently, another distinct metabolic pathway to generate bioactive sphingolipid metabolites has been described (Figure 2B). Some agents activate the de novo sphingolipid biosynthesis in the ER, i.e., daunorubicin (4), TNF-α (5), etoposide (6); some agents activate both pathways (7, 8). In any case, ceramide, sphingosine or sphinganine, and/or sphingosine-1-phosphate can also be found. More structural variations in the backbone can be found in plants, such as additional double bonds (4, 8-sphingadiene) or double bonds on C9-C19. The amid-bound fatty acids also vary in their carbon chain length (C14-C24), saturation grade (in mammalian cells mostly saturated), and α-hydroxylation. Addition of simple to rather complex headgroups forms more complex sphingolipids. In Figure 1, the structures of headgroups of sphingolipids used in our studies are shown. Altogether, variations in these components make sphingolipids the structural most diverse class of membrane lipids.

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Dietary sphingolipids in colon cancer prevention

3. SPHINGOLIPIDS IN THE DIET

3.1. Sphingolipids in foods

Although rigorous analyses of foods have yet to be performed, several foods contain significant amounts of sphingolipids (13). Especially rich sources are dairy and meat products, and eggs. Plants generally contain less sphingolipids with the exception of soybeans (Table 1). In animal products, the major sphingolipid is sphingomyelin, typically with a sphingosine backbone and saturated amide-bound fatty acids of 16, or 22-24 carbons. In plants, the prominent sphingolipid class are cerebrosides, containing a variety of different sugar headgroups (glucose, galactose, mannose), sphingoid base backbones (Figure 1) and fatty acids (often α-hydroxylated).

Sphingolipids are minor components of food, and they do not contribute significantly to the energy content of foods. Sphingolipids are also found in “fat-free” foods such as skim milk since they are localized in the membranes of the milk fat globules that partially remain in the milk after the skimming process (14). The estimated daily intake is 0.3-0.4g/day, constituting 0.01-0.02% of the diet (13). However, there are no known nutrient requirements for sphingolipids.

3.2. Digestion of dietary sphingolipids

Dietary complex sphingolipids are digested throughout the small intestine and colon by intestinal or bacterial enzymes (15-18). There is no apparent preference for a specific structure since all tested complex sphingolipids were hydrolyzed at the same rate and result...
in the generation of the bioactive metabolites ceramide and sphingosine without intermediates, suggesting a coupled hydrolysis, or cleavage by endoglycosidases (19). Ceramide and sphingosine are taken up by the cells (Figure 3) and are utilized for the synthesis of complex sphingolipids, or are degraded. There is evidence for a structure-specific fate of the sphingolipid metabolites in colonic cells since the ceramide detected after hydrolysis of complex sphingolipids contained a higher proportion of \( \text{C}_{16} \) fatty acids (19). This ceramide species has been implicated in the early phase of apoptosis (20, 21). Furthermore, although there was no difference in the digestion of sphingadiene-containing sphingolipids from corn, this sphingoid base did not accumulate in CaCo-2 colon cells to the extent that was seen with sphingosine. This was attributed to either a lower uptake or an enhanced efflux. Sphingadiene is also not a substrate for sphingosine kinase, thus, no generation of sphingosine-1-phosphate was detected. (22). Nonetheless, sphingoid bases from corn induce apoptosis in colon cancer cells (23). Therefore, the structure of the sphingolipids may affect their metabolic fate after uptake, and possibly their capacity to regulate cell growth.

The uptake of sphingolipid metabolites in the small intestine is incomplete and about 10% reach the colon where the metabolites are rapidly taken up by the colonic cells (17). Even high amounts of sphingolipids (1% of the diet or 100 times more than average estimated consumption) did not affect body weight, blood lipid levels in mature rats or their offspring (24), which may be due to the limited digestion of complex sphingolipids (25).

4. DIETARY SPHINGOLIPIDS IN COLON CANCER PREVENTION

Unrestricted growth of cancer cells is the result of hyperproliferation, the reduction of apoptosis or a combination of both events, sometimes combined with reduced differentiation. Sphingolipids are growth inhibitory, are cytotoxic to most cell lines and can induce apoptosis or differentiation. Furthermore, they have been shown to inhibit or circumvent multidrug resistance, inhibit
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**Figure 3.** Digestion and uptake of dietary sphingolipids. Complex sphingolipids in the diet are digested to ceramides and to free sphingoid bases by intestinal enzymes in the small intestine and bacteria in the colon, and taken up by the intestinal cells. Due to limited hydrolysis, excess sphingolipids are excreted in the feces.

**Table 1.** Sphingolipid content in selected foods, and estimated consumption per person per year (Vesper et al., 1999)

<table>
<thead>
<tr>
<th>Food source</th>
<th>Sphingolipid content [µmol/kg]</th>
<th>Consumption per person [µmol/year]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td>160</td>
<td>38,464</td>
</tr>
<tr>
<td>Low-fat milk</td>
<td>92</td>
<td>5,764</td>
</tr>
<tr>
<td>Cheese</td>
<td>1,260</td>
<td>15,912</td>
</tr>
<tr>
<td>Frozen dairy</td>
<td>503</td>
<td>7,042</td>
</tr>
<tr>
<td>Butter</td>
<td>460</td>
<td>1,648</td>
</tr>
<tr>
<td>Animal products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef</td>
<td>390</td>
<td>11,310</td>
</tr>
<tr>
<td>Pork</td>
<td>350</td>
<td>8,050</td>
</tr>
<tr>
<td>Chicken</td>
<td>530</td>
<td>11,660</td>
</tr>
<tr>
<td>Eggs</td>
<td>2,250</td>
<td>31,500</td>
</tr>
<tr>
<td>Vegetables and Fruits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato</td>
<td>69</td>
<td>4,116</td>
</tr>
<tr>
<td>Soybeans</td>
<td>2,410</td>
<td>N/A</td>
</tr>
<tr>
<td>Cauliflower</td>
<td>183</td>
<td>183</td>
</tr>
<tr>
<td>Peanuts</td>
<td>78</td>
<td>234</td>
</tr>
<tr>
<td>Apples</td>
<td>69</td>
<td>1,725</td>
</tr>
<tr>
<td>Bananas</td>
<td>20</td>
<td>260</td>
</tr>
</tbody>
</table>

4.1. Dietary sphingolipids prevent aberrant crypt foci in chemically induced colon cancer in CF1 mice

By feeding complex sphingolipids to mice, the bioactive metabolites are delivered directly to the colonic cells. The effects of these metabolites on normal and transformed cells *in vivo* were unknown. Therefore, we determined the effects of dietary sphingolipids on the appearance of one of the earliest visible morphological changes in colon tumorigenesis, the aberrant crypt foci (ACF). It is thought that adenomas and adenocarcinomas will develop over time from these early lesions, making ACF a widely used cost- and time effective endpoint for dietary prevention studies. Sphingolipid supplements used in these studies were about 2-10 fold above the estimated daily intake, amounts that could be achieved in the human diet.

For all ACF studies, CF1 mice were injected with 1, 2-dimethylhydrazine to induce colon tumors. Only after...
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A recent report by Exon & South (32) demonstrated that the suppression of ACF by dietary sphingolipids is not specific for mice. Rats fed sphingomyelin also developed less ACF and significantly reduced the size of ACF. Differences in the magnitude of response may be related to feeding the sphingomyelin in one dose that possibly reduces hydrolysis and enhances excretion since the digestion is dose-dependent (25).

4.2. Suppression of colon tumor formation by dietary sphingomyelin

Although ACF are widely used end-point markers in chemoprevention studies, in some instances the inhibition of ACF fails to suppress tumor formation. Therefore, we conducted a long-term study to determine if dietary sphingolipids also inhibit late stages of colon cancer. In the same study, we also evaluated if sphingomyelin supplements given before tumor initiation could suppress tumor formation even further. CF1 mice were fed 0.05% sphingomyelin in AIN 76A diet either upon arrival and throughout the study, or for 45 weeks after tumor initiation. As shown in Figure 4A, feeding sphingomyelin after tumor initiation reduced tumor incidence to 4% compared to 24% in the control (33). This validates our earlier data and demonstrates that the prevention of early stages in colon cancer by sphingolipids results in a reduction of tumor formation. However, there was no additional reduction when sphingomyelin was fed before tumor initiation. This indicates that sphingomyelin is as effective after the initial damage to the colonic cells has already occurred, and suggests a wider 'window of opportunity' for the use of orally administered sphingolipids in colon cancer prevention.

4.3. Dietary sphingolipids reduce tumor formation in Min mice

It is not known if dietary sphingolipids can prevent colon cancer in humans. However, rodent models are available that closely resemble the human disease. C57/B6JMin/+ mice (multiple intestinal neoplasia, Min mice) carry a mutation in the APC (Adenomatous Polyposis Coli) gene that also can be found in 40-80% of sporadic human colon cancers (34-35). Although these mice develop most tumors in the small intestine, they were a suitable model because sphingolipids are digested in all region of the intestinal tract (18). The diet of Min mice was supplemented with a mixture of complex sphingolipids as they appear in milk (70% sphingomyelin, 5% lactosylceramide, 7.5% glucosylceramide, and 7.5% ganglioside G03) at 0.1% of the diet beginning at the age of 35 days. Feeding the sphingolipids for 65 days reduced tumor formation by 40%. Adding ceramide to this mixture but maintaining 0.1% of the diet increased tumor suppression to 50%. This was seen in all regions of the intestinal tract including the colon (18). These studies indicate that the chemopreventive effects of dietary sphingolipids are not restricted to carcinogen-treated CF1 mice.

4.4. Effect of dietary sphingomyelin on liver cancer

Although only small portions of the orally administered sphingolipids are absorbed and transported into the body, it is possible that these amounts have a systemic effect. A recent study evaluated the effect of
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Sphingomyelin supplements on chemically induced liver cancer. Female Sprague-Dawley rats were injected with diethylnitrosamine, and fed a diet supplemented with 0.1% sphingomyelin (by weight). After 2 weeks, the number and size of enzyme-altered foci (EAF), pre-neoplastic lesions in the liver, were determined. There was a significant reduction of number and size of EAF without changes in liver morphology or growth pattern of the animals (36). These results suggest a specific effect of sphingolipids on transformation or transformed cells in amounts that do not affect normal cells and may account for the apparent lack of toxic side effects.

5. POSSIBLE MECHANISMS OF TUMOR SUPPRESSION

5.1. Regulation of proliferation and apoptosis

Since hyperproliferation and a reduced rate of apoptosis are thought to be central events in early colon carcinogenesis, a reversal of these changes may be critical to prevent tumor formation. Sphingolipids suppress cell growth and apoptosis in vitro but it is not known if the amounts of bioactive sphingolipid metabolites delivered via the diet have the same effect. Immunohistochemical analysis of the colonic tissue from carcinogen-treated CF1 mice revealed also an increase in proliferation (determined by BrdU-incorporation) and decrease in the rate of apoptosis (determined by TUNEL assay) (Figure 4B). Feeding sphingomyelin for 45 weeks reduced proliferation of colonic crypt cells to the levels of the control group that was not treated with the carcinogen, but not beyond. This reduction was also seen in the ACF studies using different sphingolipid species (19), indicating that this effect is not restricted to and specific for sphingomyelin. In addition to a reduced proliferation, the rate of apoptosis was increased by dietary sphingomyelin, but not beyond the levels of the untreated controls (Figure 4C). This shows that dietary sphingomyelin reverse the carcinogen-induced changes in CF1 mice and suggests that the 'modulation' of cell growth may be the key event in the prevention of colon cancer by dietary sphingolipids.

5.2. Regulation of β-catenin by sphingolipids

The signaling pathways leading to the 'normalization' of cell growth by dietary sphingolipids are not known. However, the identification of β-catenin, a protein especially critical in early stages of colon carcinogenesis, as a target of sphingolipids in vitro and in vitro (18) is an important step forward. As described above, dietary sphingolipids suppressed tumor formation in Min mice. The loss of the APC wild-type allele and the concomitant accumulation of cytosolic β-catenin precede tumor formation in this model. APC is located at a main ‘intersection’ of intracellular metabolism, regulating a multitude of processes. Important for colon cancer is the regulation of β-catenin degradation by APC. β-catenin is a cell adhesion protein that connects E-cadherin to the actin cytoskeleton (37-39). It also functions as a signaling molecule in developmental systems (40-41), and in the response of cells to growth stimulation (i.e. Wnt/Wingless pathway, epidermal growth factor and hepatocyte growth factor receptor activation; 37, 42-43). APC mutations result in the stabilization of β-catenin with subsequent accumulation in the cytosol, translocation to the nucleus and activation of transcription factors (see review 44). This activates the transcription of genes that are correlated to unrestricted growth in colon cancer.

Immunohistochemical analyses of the intestinal tissue from Min mice showed a high expression of β-catenin in the cytosol of intestinal sections from control mice. However, Min mice that were fed sphingolipid supplements in their diet and that had developed only a small number of tumors displayed mostly membrane-associated β-catenin. This is its normal localization, found in the genetic background mice, identifying β-catenin as a target of dietary sphingolipids (18). The modulation of β-catenin localization was also demonstrated in vitro, using human colon cancer cell lines carrying APC mutations. It is not known if the regulation of β-catenin is the central event in tumor prevention by dietary sphingolipids but it is clearly an important event, and may directly contribute to the anti-colon cancer effects of sphingolipids. Furthermore, APC mutations are not only important in human colon cancer (45) but in a growing list of cancer in other organs such as esophagus, breast, liver and stomach, and the use of orally or otherwise administered sphingolipids may also be beneficial in the prevention of these cancers.

6. PERSPECTIVE

Dietary sphingolipids significantly reduce colon cancer in mice. Although some epidemiological studies suggest an association of consumption of dairy products with a decrease in colon cancer risk (46), it is still unknown if dietary sphingolipids would also have a beneficial effect in humans. In contrast to many conventional drugs, dietary or other orally administered sphingolipids do not cause any apparent toxic side effects, and doses that are effective in mice can be achieved in humans. However, much more research is necessary to determine the signaling pathways dietary sphingolipids activate/inactivate to prevent tumor formation in order to develop a strategy to prevent colon cancer with orally administered sphingolipids, and establish molecular markers to predict its efficacy. Moreover, the effective concentrations and conditions for chemoprevention may be different from the use of sphingolipids as chemotherapeutic agents to induce apoptosis in cancer cells. The clear advantage of orally administered sphingolipids is the lack of deleterious side effects. This is expected to allow for an unlimited treatment period, to increase the compliance of patients, or people with a high risk of developing colon cancer, with the treatment and to enhance their quality of life during treatment.

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*Nutr* 124, 615-620 (1994)


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