Suppression of colitis-driven colon cancer in mice by a novel small molecule inhibitor of sphingosine kinase

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Sphingolipid metabolism is driven by inflammatory cytokines. These cascade of events include the activation of sphingosine kinase (SK), and subsequent production of the mitogenic and proinflammatory lipid sphingosine 1-phosphate (S1P). Overall, S1P is one of the crucial components in inflammation, making SK an excellent target for the development of new anti-inflammatory drugs. We have recently shown that SK inhibitors suppress colitis and hypothesize here that the novel SK inhibitor, ABC294640, prevents the development of colon cancer. In an azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model, there was a dose-dependent decrease in tumor incidence with SK inhibitor treatment. The tumor incidence (number of animals with tumors per group) in the vehicle, ABC294640 (20 mg/kg) and ABC294640 (50 mg/kg) groups were 80, 40 and 30%, respectively. Tumor multiplicity (number of tumors per animal) also decreased from 2.1 ± 0.23 tumors per animal in the AOM + DSS + vehicle group to 1.2 ± 0.0 tumors per animal in the AOM + DSS + ABC294640 (20 mg/kg) and to 0.8 ± 0.4 tumors per animal in the AOM + DSS + ABC294640 (50 mg/kg) group. Importantly, with ABC294640, there were no observed toxic side effects. To explore mechanisms, we isolated cells from the colon (CD45-; representing primarily colon epithelial cells) and (CD45+, representing primarily colon inflammatory cells) then measured known targets of SK that control cell survival. Results are consistent with the hypothesis that the inhibition of SK activity by our novel SK inhibitor modulates key pathways involved in cell survival and may be a viable treatment strategy for the chemoprevention colitis-driven colon cancer.

Introduction

Inflammatory bowel disease (IBD) is a group of disorders characterized by pathological inflammation of the lower intestine. Crohn’s Disease and Ulcerative Colitis are the most common forms. The roles of immune cells and inflammatory cytokines in the pathogenesis of IBD are well established. Activation of immune responses in IBD results in the local influx of mast cells, monocytes, macrophages and neutrophils, which produces the clinical manifestations of the diseases (1). This is accompanied by dramatic increases in tumor necrosis factor-alpha (TNFα) (2), which has a primary role in mediating both experimental and clinical IBD. Additionally, IBD is a high colon cancer risk disease. Cancers appear to arise from genetic alterations in cancer-related genes (oncogenes and suppressor genes), as well as an overall loss of genomic stability. The etiological and tissue microenvironment factors that spawn colitis and progression to colon cancer are still unclear. So far, this has resulted in limited success for prevention of colitis and colon cancer associated with this disease, and new molecular targets for their prevention and/or treatment are needed.

The mechanisms and effects of the sphingolipid interconversion have been the subjects of a growing body of scientific investigation. Sphingomyelin is a structural component of cellular membranes and is a precursor for the potent bioactive lipids ceramide and sphingosine 1-phosphate (S1P). A ceramide/S1P rheostat has been hypothesized to determine the fate of the cell, such that the relative cellular concentrations of ceramide and S1P determine whether a cell proliferates or undergoes apoptosis. Ceramide is produced by the hydrolysis of sphingomyelin in response to inflammatory stresses, including TNFα, which is a key target in colitis (3,4). Ceramide induces apoptosis in tumor cells (5) and can be further hydrolyzed by the action of ceramidase to produce sphingosine (Sph). Sph is then rapidly phosphorylated by sphingosine kinase (SK) to produce S1P. Ceramidase and SK are also activated by cytokines and growth factors, leading to rapid increases in the intracellular levels of S1P and depletion of ceramide levels. This promotes cell proliferation and inhibits apoptosis, and deregulation of apoptosis in phagocytes is an important component of the chronic inflammatory state in IBDs.

S1P also has several important effects on immune function. Platelets, monocytes and mast cells secrete S1P upon activation, promoting inflammatory cascades in damaged tissue (6). SK activation is required for the signaling responses since the ability of TNFα to induce adhesion molecule expression via activation of nuclear factor-kappaB is mimicked by S1P and blocked by the SK inhibitor dimethylsphingosine (3). Similarly, S1P mimics the ability of TNFα to induce COX-2 expression and prostaglandin E2 synthesis; and knock down of SK blocks these responses to TNFα but not S1P (7). In intestinal endothelial cells, S1P activates nuclear factor-kappaB thereby inducing the expression of adhesion molecules, COX-2 resulting in prostaglandin E2 synthesis and nitric oxide synthase producing nitric oxide. Together, these chemotactants and the adhesion molecules promote neutrophil infiltration into the mucosa. SK has been directly demonstrated to play a significant role in the recruitment and function of these cells (8,9). At the same time, S1P activates neutrophils resulting in the release of oxygen free radicals that further inflame and destroy epithelial tissues. Overall, it is clear that S1P is critical in inflammation, making SK an excellent target for the development of new anti-inflammatory/chemopreventive drugs. Here, we confirm that a novel small molecule SK inhibitor [ABC294640, (10)] can prevent colitis (11) and show for the first time that it also suppresses colon cancer associated with colitis in a dose–response manner.

Materials and methods

Animals

All mice (C57BL/6) used were 8–12 weeks of age. They were housed in pathogen-free rooms in filter-topped cages at the Laboratory Animal Facility at Graduate Science Research Center, University of South Carolina. This facility is accredited by Association for Assessment and Accreditation of Laboratory Animal Care-International, is registered with the United States Department of Agriculture (56-R-003) and operates in accordance with Federal (Guide for the Use and Care of Laboratory Animals, Animal Welfare Act), State and local laws and regulations. All mice were used in accordance with
protocols approved by the Institutional Animal Care and Use Committees of University of South Carolina. Mice received food and water ad libitum.

ABC294640

The SK inhibitor, ABC294640 whose pharmacokinetics have been described previously (10), was provided by Apoee Biotechnology Corporation (Hershey, PA) (11).

AOM/DSS-induced colon cancer model

We followed a modified protocol outlined recently by the Wirz et al. (12) (supplementary Figure 1A is available at Carcinogenesis Online). The supplementary Figure 1B (available at Carcinogenesis Online) also outlines the treatment groups for this study. Briefly, these are as follows: Group 1, vehicle (PEG400 alone); Group 2, azoxymethane (AOM) + PEG400; Group 3, Dextran sulfate sodium (DSS) + PEG400; Group 4, ABC294640 (50 mg/kg) alone; Group 5, AOM + ABC294640 (50 mg/kg); Group 6, AOM + DSS + vehicle (PEG400); Group 7, AOM + DSS + ABC294640 (20 mg/kg) and Group 8, AOM + DSS + ABC294640 (50 mg/kg). Ten to twelve weeks old C57BL/6 male and female mice were randomized, weighed and given a single intraperitoneal injection of AOM (10 mg/kg; Sigma Chemical Co., St. Louis, MO) or vehicle on Day 1. One week later, daily intraperitoneal administration of either high (50 mg/kg) or low (20 mg/kg) doses of ABC294640 or vehicle (PEG400) was initiated as summarized in supplementary Figure 1A, available at Carcinogenesis Online. As well, animals received either 1% DSS (Molecular Weight 36 000–50 000; MP Biomedicals, Solon, OH) or normal drinking water. Chronic IBD was induced with cyclical DSS treatments, consisting of 7 days of 1% DSS followed by 14 days of normal water for a total of three cycles. The SK inhibitor (or PEG400 vehicle control) was administered 5 days per week until the end of the experiment on Day 70. The AOM-only groups were included to determine whether ABC294640 administered 5 days per week until the end of the experiment on Day 70.

Statistics

A chi-square contingency table analysis was done on the AOM + DSS and AOM + DSS + SK inhibitor groups to determine if there is a statistically significant difference in their inflammation scores and Sph levels. Tumor incidence was examined using a Fisher’s exact test, which is equivalent to a test for binomial proportions. Because the usual assumptions underlying the analysis of variance test are not satisfied, in assessing the significance of the F-statistic value from the analysis of variance table, instead of using the F-distribution, we used a permutation distribution to examine tumor multiplicity. Tumor sizes were divided into <5 mm² and 5–50 mm², and the analysis of variance and Kruskal–Wallis test were carried out for significant differences between the groups. The P-value chosen for significance in this study was 0.05.

Results

The small molecule SK inhibitor, ABC294640, suppresses colitis

Recently, it has been shown that SK−/− mice had significantly less DSS-induced colonic inflammation than wild-type counterparts (13). In addition, we have shown that the pharmacological SK inhibitor, ABC294640, also inhibits DSS-induced colitis (11). To confirm ABC294640 was working to inhibit colon inflammation in this experiment, five mice were examined and inflammation scores were recorded. Figure 1A shows that AOM-injected mice consuming DSS had significantly elevated levels of colon inflammation (P = 0.005) and that administration of the SK inhibitor significantly decreased the inflammatory score (P = 0.0136 compared with the AOM + DSS + vehicle group).

As a separate measure of gastrointestinal and overall health, we also measured colon length (Figure 1B), body weight change (Figure 1C) and white blood cell counts (Figure 1D). Mouse colon length shrinks with stress, inflammation and ulceration (14). Results indicate that compared with the colon lengths of the control (AOM + PEG400)-treated group (8.3 ± 0.16 cm), the length was significantly (P = 0.0000023) reduced in the AOM + DSS group (5.2 ± 0.25 cm). AOM + DSS mice treated with the SK inhibitor (ABC294640, 50 mg/kg) had statistically longer colons (7.1 ± 0.23 cm) compared with those of the AOM + DSS group (P = 0.000074). Similarly, unhealthy mice lose weight during the course of the experiment. To that end, over the 15 day course of this intermediate end point experiment, the AOM + DSS group lost an average of 0.87 ± 0.18 g, whereas both the non-AOM-treated and the AOM + DSS + ABC294640 (50 mg/kg) gained an average of 1.34 ± 0.36 and 0.67 ± 0.27 g, respectively. These values were significantly different from the AOM + DSS group (P < 0.05). Finally, a complete white blood cell count was carried out to monitor systemic inflammation. The PEG400-treated group had an average total circulating white blood cell count (×10⁸ cells/mL) of 9.5 ± 0.7. The AOM + DSS...
and AOM + DSS + ABC294640 (50 mg/kg) groups had an average count of 12.7 ± 3 and 9.1 ± 1.4, respectively, indicating less systemic stress with injection of 50 mg/kg ABC294640. The red blood cell counts were 9.66 ± 0.8, 9.63 ± 0.6 and 8.34 ± 1.0 for the PEG400, AOM + DSS and AOM + DSS + ABC294640 (50 mg/kg) groups, respectively. These values, however, were not statistically significant (P > 0.05).

SK activity in target colons

As a measure of the effects of SK inhibitors in this animal model, sphingolipid profiles were assayed in the colons of animals using liquid chromatography-tandem mass spectroscopy. This technique allows the examination of correlations between biologic activity and changes in sphingolipid levels in animals treated with SK inhibitors. While S1P levels are the most direct indicators of SK activity, the levels of S1P in the colon samples were below the detection limit. However, the substrate Sph levels should increase when SK is inhibited, and in fact, Sph levels were significantly (P < 0.05) higher in colons from AOM + DSS + ABC294640 mice compared with AOM + DSS + vehicle-treated controls in both the Days 15 and 70 samples (Figure 2).

The small molecule SK inhibitor, ABC294640, suppresses AOM + DSS-induced macroscopic lesions

On experimental Day 70 (supplementary Figure 1 is available at Carcinogenesis Online), colons were harvested and stained with methyl blue to examine macroscopic lesions. A total of 12/13 (92.3%) mice treated with AOM + DSS + vehicle (PEG400), but 0/10 (0%) in vehicle control only (no AOM) developed macroscopic lesions in the colon (Table I). There was a dose-dependent decrease in the incidence of macroscopic lesions with the SK inhibitor treatment, such that 7/10 (70%) of mice developed macroscopic lesions in the AOM + DSS + ABC294640 (20 mg/kg) group and 5/10 (50%) developed macroscopic lesions in the AOM + DSS + ABC294640 (50 mg/kg) group. Although the difference between the AOM + DSS + vehicle (92.3% incidence) and AOM + DSS + ABC294640 (20 mg/kg) (70% incidence) was not statistically significant (P = 0.28); significance was achieved between the AOM + DSS + vehicle (92.3% incidence) and AOM + DSS + ABC294640 (50 mg/kg) (50% incidence) groups (P = 0.05).

The macroscopic lesion multiplicity (number of macroscopic lesions per animal) was also decreased by treatment with the SK inhibitor (Table I). The total number of macroscopic lesions in the AOM + DSS + vehicle (PEG400) was 64, the total number of macroscopic lesions in the ABC294640 (20 mg/kg) group was 24, and the total number of macroscopic lesions in the ABC294640 (50 mg/kg) group was 16. Permutation distribution analysis found similar results to that of macroscopic lesions. Although the difference between the AOM + DSS + vehicle (PEG400) (4.85 ± 1.1 macroscopic lesions per animal) and AOM + DSS + ABC294640 (20 mg/kg) (2.4 ± 1.0 macroscopic lesions per animal) was not statistically significant (P = 0.14); significance was achieved between the AOM + DSS + vehicle (92.3% incidence) and AOM + DSS + ABC294640 (50 mg/kg) (50% incidence) groups (P = 0.03). There were two macroscopic lesions in one animal from the AOM + ABC294640 (50 mg/kg) and one macroscopic lesion in one animal from the DSS + (PEG400)-treated groups. All other groups were tumor-free (Table I).

Another trend was that the tumor surface area is reduced with increasing doses of ABC294640 (Table I). Tumors (32%) in the AOM + DSS group were >3 mm² without ABC294640, but only 25 and 13%
of tumors were that large in the 20 and 50 mg/kg ABC294640 groups, respectively. This trend, however, was not statistically significant. Supplementary Figure 2 (available at Carcinogenesis Online) shows Table I data in graphical form, as well as representative photographs of colons from each group. The arrows indicate a colonic neoplasm. Figure 3 shows representative hematoxylin- and eosin-stained photomicrographs of tissues on Days 15 and 70.

The small molecule SK inhibitor, ABC294640, suppresses microscopic colon cancer associated with colitis

Because many lesions are not macroscopically evident, and because some growths are not necessarily cancerous, we sectioned each colon for microscopic histology studies. The differences between Tables I and II reflect that this statement is true. As shown in Table II, tumor incidence of mice treated with AOM + DSS + vehicle (PEG400) was 80%, and a dose-dependent decrease in tumor incidence occurred with SK inhibitor treatment. A total of 4/10 (40%) mice developed tumors in the AOM + DSS + ABC294640 (20 mg/kg) group and 3/10 (30%) developed tumors in the AOM + DSS + ABC294640 (50 mg/kg) group. Both results were statistically significant (P < 0.05).

Tumor multiplicity (number of tumors per animal) also decreased with SK inhibitor treatment (Table II). The difference between the AOM + DSS + vehicle (PEG400) (2.1 ± 0.23 tumors per animal) and AOM + DSS + ABC294640 (20 mg/kg) (1.2 ± 0 tumors per animal) was statistically significant (P < 0.05); significance was also achieved between the AOM + DSS + vehicle (PEG400) (2.1 ± 0.23 tumors per animal) and AOM + DSS + ABC294640 (50 mg/kg) (0.8 ± 0.4 tumors per animal) groups (P < 0.05). There were two tumors in one animal from the DSS + vehicle (PEG400) control group. All other groups were tumor-free (Table I).

Fig. 2. Effects of the SK inhibitor ABC294640 (50 mg/kg dose) on Sph levels in colons of animals in the AOM + DSS mouse model of colitis. Colon samples from mice described in Table I were extracted and assayed for levels of Sph by liquid chromatography-tandem mass spectroscopy as described in the ‘Materials and Methods’ section. Values represent the mean ± standard error for five samples per group.

Fig. 3. Protective effect of SK inhibitor ABC294640 on AOM/DSS colitis-induced epithelial damage. Representative microphotographs for AOM + Vehicle (A), AOM + DSS (B) and AOM + DSS + ABC294640 (50 mg/kg) (C) groups at Days 15 (left panels) and 70 (right panels).
Sphingosine Kinase and Colon Cancer

Table II. Histological tumor incidence and multiplicity in mice treated with AOM/DSS ± ABC294640

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Percentage of animals with colon tumors (incidence)</th>
<th>Number of tumors per animal (multiplicity) mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (PEG400)</td>
<td>10</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>AOM + vehicle (PEG400)</td>
<td>10</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>DSS + vehicle (PEG400)</td>
<td>10</td>
<td>10.0</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Vehicle + ABC294640 (50 mg/kg)</td>
<td>10</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>AOM + ABC294640 (50 mg/kg)</td>
<td>10</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>AOM + DSS + vehicle (PEG400)</td>
<td>10</td>
<td>80.0</td>
<td>2.1 ± 0.23</td>
</tr>
<tr>
<td>AOM + DSS + ABC294640 (20 mg/kg)</td>
<td>10</td>
<td>40.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AOM + DSS + ABC294640 (50 mg/kg)</td>
<td>10</td>
<td>30.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.8 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Indicates significant difference from AOM + DSS-treated group (see Materials and Methods for statistics).

Histologically, we found that all growths were adenomas. In the AOM + DSS + vehicle (PEG400)-treated group, 42% of the lesions were adenomas with low-grade dysplasia and 47% were adenomas with high-grade dysplasia. The remaining lesions were carcinoma in situ. In the AOM + DSS + ABC294640 (20 mg/kg)-treated group, similarly, 42% of the lesions were adenomas with low-grade dysplasia and 25% were adenomas with high-grade dysplasia and the remaining carcinoma in situ. In the AOM + DSS + ABC294640 (50 mg/kg)-treated group, similarly, 26% of the lesions were adenomas with low-grade dysplasia and 47% were adenomas with high-grade dysplasia. The remaining were carcinoma in situ. Interestingly, three mice from this latter group [AOM + DSS + ABC294640 (50 mg/kg)] had areas of significant squamous metaplasia.

The small molecule SK inhibitor, ABC294640, targets both epithelial and inflammatory cells for death pathways

We and others have recently characterized some of the targets of SKs, which include regulators of cell survival (15,16). Beclin-1, a key protein regulating the autophagy pathway, is a tumor suppressor and an important component regulating cell survival. Other targets of SKs include the AKT pathway and the ERK1/2 pathway. These proteins also promote cell survival following phosphorylation (17), which is elevated in both inflammatory cells and epithelial cells in colitis (18–22). To this end, we wanted to better understand the mechanism by which ABC294640 inhibits colon cancer associated with colitis. We therefore isolated CD45<sup>+</sup> inflammatory cells and CD45<sup>-</sup>epithelial cells in a pre-cancerous stage (Day 15 of 70) and probed the cells for Beclin-1, AKT phosphorylation and ERK1/2 phosphorylation. Figure 4 shows that Beclin-1 is suppressed by DSS + AOM in epithelial cells, whereas the addition of ABC294640 elevated Beclin-1. Phosphorylated AKT and ERK1/2 were elevated in epithelial cells with AOM + DSS treatment and suppressed by treatment of the mice with ABC294640. Similar results were found in inflammatory cells.

Discussion

Colitis is a dynamic disease associated with hyperactive immune cells and increased colon cancer risk. Based on the knowledge that S1P can potentiate inflammation by inhibiting the apoptosis of inflammatory cells, we have recently shown that SK inhibitors suppress colitis in a mouse model (11). Here, we have extended this data to show that the SK inhibitor, ABC294640, not only inhibits colitis (Figure 1), but also inhibits colon cancer associated with colitis (Tables I and II). To investigate any immunomodulatory effects, we also performed blood counts between the control and SK inhibitor group. Mechanistically, we have also shown that SK was inhibited in vivo, consistent with the understanding that ABC294640 was responsible for the prevention of cancer. Additionally, we found that Beclin-1 was suppressed, and AKT ERK1/2 were phosphorylated in the colitis model, and these parameters are largely normalized by treatment with ABC294640. The understanding that these are targets of SK (15,16) and finding that these survival pathways occurred in colon epithelial cells not only gives insight into a possible mechanism of colon cancer development in the AOM/DSS models, but also the prevention of colitis-driven colon cancer by ABC294640.

Kohno et al. (23) recently showed adenoma size but not incidence was dramatically reduced in Apc Min<sup>+/−</sup> SK<sup>−/−</sup> mice. Although this is consistent with our finding that the inhibition of SK can protect against colon cancer, the pathology and the molecular characteristics of sporadic colon cancer are different than colitis-driven colon cancer. Our study is in agreement, however, with another recent study by Kawamori et al. (24). Using a similar AOM + DSS model, they showed that SK1-knockout mice had reduced tumor incidence and multiplicity. We should note that ABC294640 is a stronger inhibitor of SK-2 (10,15), adding to the novelty and potential clinical relevance of our findings.

The mechanism of inhibition of colon cancer associated with colitis by ABC294640 is most probably associated with its ability to inhibit inflammation. However, it is possible that there are protective effects directly on the colon epithelial cells, as demonstrated by our findings presented in Figure 4. Specifically, it appears Beclin-1, AKT and/or
ERK1/2 are candidate molecules/pathways that are targeted by ABC294640 to inhibit colon carcinogenesis. Others have found that transfection of SK into NIH3T3 fibroblasts is sufficient to promote foci formation and cell growth in soft-agar and to allow these cells to form tumors in NOD/SCID mice. Additionally, SK inhibition by transfection with a dominant-negative SK mutant or by treatment of cells with the non-specific SK inhibitor N-erythro-4,5-dimethylsphingosine blocks transformation mediated by oncogenic H-Ras (25). As abnormal activation of Ras frequently occurs in cancer, these findings suggest a significant role of SK in this disease. SK has also been linked to estrogen signaling (26) and estrogenic H-Ras (25). As abnormal activation of Ras frequently occurs in many sufferers of IBD.

ERK1/2 appears to be the most direct mitogenic messenger, inhibition of its activity has been linked to estrogen signaling (26) and estrogen-responsive H-Ras (25). As abnormal activation of Ras frequently occurs in many sufferers of IBD, SK has also been linked to estrogen signaling (26) and estrogenic H-Ras (25). As abnormal activation of Ras frequently occurs in many sufferers of IBD.

References


Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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