Enhanced Colonic Tumorigenesis in Alkaline Sphingomyelinase (NPP7) Knockout Mice

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Abstract

Intestinal alkaline sphingomyelinase (alk-SMase) generates ceramide and inactivates platelet-activating factor (PAF) and was previously suggested to have anticancer properties. The direct evidence is still lacking. We studied colonic tumorigenesis in alk-SMase knockout (KO) mice. Formation of aberrant crypt foci (ACF) was examined after azoxymethane (AOM) injection. Tumor was induced by AOM alone, a conventional AOM/dextran sulfate sodium (DSS) treatment, and an enhanced AOM/DSS method. β-Catenin was determined by immunohistochemistry, PAF levels by ELISA, and sphingomyelin metabolites by mass spectrometry. Without treatment, spontaneous tumorigenesis was not identified but the intestinal mucosa appeared thicker in KO than in wild-type (WT) littermates. AOM alone induced more ACF in KO mice but no tumors 28 weeks after injection. However, combination of AOM/DSS treatments induced colonic tumors and the incidence was significantly higher in KO than in WT mice. By the enhanced AOM/DSS method, tumor number per mouse increased 4.5 times and tumor size 1.8 times in KO compared with WT mice. Although all tumors were adenomas in WT mice, 32% were adenocarcinomas in KO mice. Compared with WT mice, cytosol expression of β-catenin was significantly increased and nuclear translocation in tumors was more pronounced in KO mice. Lipid analysis showed decreased ceramide in small intestine and increased sphingosine-1-phosphate (S1P) in both small intestine and colon in nontreated KO mice. PAF levels in feces were significantly higher in the KO mice after AOM/DSS treatment. In conclusion, lack of alk-SMase markedly increases AOM/DSS–induced colonic tumorigenesis associated with decreased ceramide and increased S1P and PAF levels. Mol Cancer Ther; 14(1): 259–67. ©2014 AACR.

Introduction

Metabolism of sphingolipids has important implications in many diseases, including inflammatory bowel disease and colon cancer (1). Sphingomyelin is a major type of sphingolipids existing in all eukaryotic cell membranes and dietary products such as milk, egg, and meat (2, 3). Hydrolysis of sphingomyelin generates ceramide that can inhibit cell proliferation and induce differentiation and apoptosis via numerous signaling pathways (4). Ceramide can be degraded to sphingosine that can in turn form sphingosine-1-phosphate (S1P) by sphingosine kinase (SphK) or be converted to ceramide-1-phosphate (C1P) by ceramide kinase.

In contrast with ceramide, S1P and C1P have stimulatory effects on cell proliferation, cell migration, and angiogenesis (5, 6). The balance of ceramide and S1P is a key factor determining cell fate, thus having important implications in tumorigenesis (7).

It has been shown that feeding animals with sphingomyelin or ceramide analogues attenuated colonic tumorigenesis (8, 9). The key enzyme in the gut that hydrolyses sphingomyelin to ceramide is alkaline sphingomyelinase (alk-SMase; ref. 10), which was discovered, purified, and cloned in our group (11, 12). Cloning studies reveal that alk-SMase shares no structural similarities with other SMases, but belongs to the nucleotide pyrophosphatase phosphodiesterase (NPP) family and is now also called NPP7 (11, 13, 14). However, NPP7 is inactive toward nucleotide phosphates esters but has specific activities against phospholipids containing a phosphocholine moiety, including sphingomyelin, platelet-activating factor (PAF), and lysophosphatidylcholine (13, 15). Hydrolyses of these substrates result in the increased ceramide formation, inhibited proinflammatory effects of PAF, and reduced formation of lysophosphatidic acid. All have implications in carcinogenesis (16).

In patients, alk-SMase activity is decreased by 25% in chronic colitis and by 75% in colon cancer (17, 18). Low activity has also been found in the feces from patients with colon cancer (19) and in bile of patients with hepatobiliary cancers (20). An alternative splicing form of alk-SMase has been detected in both colonic and hepatic cancer cells (21, 22). The aberrant form has impaired substrate-binding site rendering the enzyme inactive. All these findings lead to a hypothesis that alk-SMase is an anticancer factor in the gut. Direct evidence is, however, still lacking. We here examined the tumorigenesis in alk-SMase knockout (KO) mice generated in our group by the Cre-LoxP system (10) and show that
lack of alk-SMase significantly enhanced colon cancer susceptibility induced by carcinogens and inflammation.

**Materials and Methods**

**Materials**

Azoxymethane (AOM), anti-β-catenin antibody, and second antibodies used were purchased from Sigma-Aldrich, antimiNPP7 antibody from R&D Systems, and dextran sulfate sodium (DSS) with M.W. 36,000–50,000 from MP Biomedicals. The Genomic DNA Extraction Kit was from Fermentas and the PAF Assay Kit from Antibodies-Online, Aachen Germany. Standards of sphingomyelin (d18:1/16:0 and d18:0/16:0), ceramide (d18:1/17:0, d18:1/16:0 and d18:0/16:0), and S1P (18:1) were purchased from Avanti Polar Lipids.

**Animals**

Alk-SMase KO mice were generated by the Cre-LoxP system as described previously (10). In brief, two loxP sites are inserted to flank the exon 2 of ENPP7 located on chromosome 11 (Ensembl Gene ID:ENSMUSG 00000046697). Deletion of the floxed region upon Cre recombinase reaction causes a frame-shift, which creates a novel stop codon that stops the translation at early stage. The KO mice were maintained on C57BL/6 background and the mice were backcrossed eight times to get an inbred strain. Unless specified elsewhere age and sex matched C57BL/6 WT (wild-type) mice were purchased from Mollegaard; same source for backcross of the mice as described previously (10). The animals were fed standard pellets with free access to water. The ethics permit for generating and using WT and KO mice in the study was issued by the Malmö/Lund Ethics Committee.

**Histology and histopathology examinations of the intestinal tract**

The tissue samples of colon and small intestine were fixed in 4% paraformaldehyde in 0.1 mol/L PBS pH 7.2 overnight and then stored in 70% ethanol. The samples were then dehydrated and embedded in paraffin. Three sections from each sample were cut, dewaxed, and stained in hematoxylin and eosin. The mucosal thickness of small intestine and colon was measured from the longitudinal sections by Image Scope software, which was performed in four littermates (2 WT and 2 KO). Only the intact villi or crypt was taken into account. In tumorigenesis studies, the tumors identified were excised together with the surrounding tissues and handled similarly as above for histopathology examination.

**Study protocols in tumorigenesis**

The studies were started in mice at the age of 8 to 10 weeks. Four study protocols were used to examine the tumorigenesis.

Study A examines aberrant crypt foci (ACF) formation according to Bird and colleagues (23). In brief, 3 WT and 3 KO mice were s.c. injected with AOM (12 mg/kg), once a week for 2 weeks. Four weeks after the last injection, the colon was stained with methylene blue and the number and the size of ACF were counted under a dissecting microscope by a person unaware of the sample source.

Study B examines tumorigenesis induced by AOM alone. Ten WT and 10 KO mice were injected with AOM as in Study A. The tumorigenesis was examined 28 weeks after the last injection.

Because in Study B, tumorigenesis was not identified, a conventional combination of AOM and DSS treatments was used (Study C; refs. 24, 25). Ten WT and 10 KO mice were injected with AOM (10 mg/kg) once. One week later, the animals were given 1% DSS in the drinking water for 1 week followed by 2 weeks of tap water. The DSS treatment was repeated for additional 2 cycles. Two weeks after the last cycle, the tumorigenesis was examined.

Study D examines tumorigenesis with a long-term enhanced method that combined the treatments in Study B and C. Ten WT and 10 KO mice were injected with AOM twice as in Study B. Twenty-one weeks later, the mice received an additional AOM injection followed by three cycles of DSS treatment as in Study C. The tumorigenesis was examined 2 weeks after the last DSS cycle. In Study B, C, and D, the body weights of the mice were measured at least once a week.

![Figure 1](https://example.com/figure1.png)

Alk-SMase expression and morphologic changes of the colon in the WT and KO mice. A, cellular location of alk-SMase on the apical surface of the villi in WT mice, and disappearance in the KO mice (B). C and D, representative micrographs showing the increased colonic thickness in KO mice compared with WT mice, F, aberrant crypt with hyperchromatism and nuclear crowding (pointed by arrows) in KO mice compared with the WT mice (E), after AOM treatment in Study B; bar, 20 μm.
Upon examination, the animals were anesthetized with xylazine/ketamine. The small intestine and colon were removed and cut opened. The number of tumors appearing was counted by the naked eyes and rechecked under a dissecting microscope (×40). The length and the width of each tumor were measured by a digital micro ruler. The tumor area was calculated by multiplying length and width. The tumors and the surrounding tissues were then excised, fixed, and stained for pathologic examination. The signs of dysplasia and tumorigenesis were evaluated regarding cellular morphology, nucleoplasm ratio, and mitoses.

Immunohistochemistry studies for expression of β-catenin

IHC analysis of β-catenin was performed in both nontreated WT (n = 3) and KO (n = 3) mice as well as in AOM/DSS-treated WT (n = 3) and KO (n = 5) mice in Study D. Longitudinal sections of colon were used. Paraffin sections (5 μm) were deparaffinized, hydrated, and subjected to antigen retrieval by boiling in citrate buffer (0.01 mol/L pH 6) for 2 × 8 minutes. Sections were cooled and washed in PBS containing 0.25% Triton X100 (PBST) before being incubated overnight at 4°C with a polyclonal β-catenin antibody diluted (1:8,000) in PBST containing 0.25% BSA. β-Catenin was visualized either with fluorescein (Z0196 Dako) or Alexa Flour 594 conjugated IgG antibodies (Jackson ImmunoResearch). Secondary antibodies were mixed and incubated 1 hour at room temperature. Hoechst (Life Technologies) cell nuclei counter staining was performed according to the manufacturer’s protocol. Mounting was in PBS:glycerol 1:1 followed by fluorescence microscopy (Olympus BX43, LRI) with appropriate filter setting. IHC of alk-SMase in the intestinal mucosa of WT and KO mice was performed as above using polyclonal anti-NPP7 as first antibody and anti-goat IgG as second antibody.

Assay of sphingolipids in the intestinal mucosa by MS

Sphingomyelin metabolites were determined in 6 WT and 6 KO nontreated mice. After sacrificing, the intestinal tract was removed and intestinal content and feces were washed out with ice-cold
PBS buffer. The tissues were frozen immediately in liquid nitrogen. The phospholipids and sphingolipids were extracted as described previously (26). In brief, the frozen tissues were homogenized and 50 μL of homogenate was mixed with 950 μL methanol containing internal standards. After lipid extraction, 800 μL of supernatant was directly used for S1P analysis in negative ion MRM mode, and sphingomyelin and ceramide, in positive ion MRM mode. The analyses were performed using the API 4500 QTRAP mass spectrometer (Applied Biosystems/MDS SCIEX). Both nebulizer and desolvation gases were nitrogen. Samples were loaded through an LC system with an auto sampler. Typical operating parameters were as follows: curtain gas 25, collision gas medium, ion source gas 1 (GS1) 45, and ion source gas 2 (GS2) 50. Electrospray voltage with positive ion MRM mode was 5500 and that with negative ion mode, −4500. The temperature of the heater was 500°C.

Measurement of PAF
After the AOM and DSS treatments in Study C, animals were sacrificed and tumorigenesis examined. Fecal samples were collected from the colon, dried under nitrogen gas, and weighed. The dried feces were suspended in saline, centrifuged at 10,000 rcf at 4°C for 10 minutes and the supernatant was collected. The whole colonic mucosa was scraped and lysed as described previously (10), followed by centrifugation at 10,000 rcf at 4°C for 10 minutes. The levels of PAF in feces and mucosal lysates were determined by an ELISA kit according to the manufacturer's protocol and the values were adjusted with the fecal weight or protein levels of the samples.

Statistical analysis
The results are expressed as mean ± SE. Statistical analyses were performed by the nonpaired Student t test and a P value of <0.05 was considered statistical significant.

Results
Observation of intestinal morphology and spontaneous tumorigenesis in the KO mice
The main genotypic and phenotypic changes in the KO mice have been reported previously (10). Alk-SMase activity was not detectable in intestinal mucosa, content, and feces in these KO mice.
mice. The animals look normal and no significant changes were found for body weight, food intake, intestinal movement, small intestine and colon length, and fertility. In the present study, we further assessed alk-SMase expression in WT and the KO mice by IHC. As shown in the top in Fig. 1, intense alk-SMase immunoreactivity is located at the apical surface of the columnar epithelium covering the villi in WT mice. No alk-SMase labeling is detected in the KO mice. Intestinal histology was compared in 4 littersmates (2 WT and 2 KO) and the mucosal architecture of the KO mice was largely normal. However, the colonic mucosa in KO mice increased by 22% (260.5 ± C6 3.6 vs. 214.0 ± C6 4.6 mm, P < 0.0001, n = 62 for KO and 56 for WT), and the small intestinal mucosa increased by 9% (463.6 ± C6 3.9 vs. 424.5 ± C6 7.0 mm, P < 0.0001, n = 36 for both groups) compared with WT mice. Representative micrographs of colonic thickness are shown in the middle (Fig. 1C and D). Spontaneous tumorigenesis was examined in 4, 5, and 3 KO mice at the age of 10, 12, and 17 months, respectively. No tumors were identified either in the small intestine or colon.

Study A and B: effects of AOM alone on ACF formation and colonic tumorigenesis

Four weeks after injection of AOM the ACF number per mouse in 3 WT mice were 15.3 ± 2.23 and that in KO mice, 24.0 ± 1.5 (P < 0.05). However, when examined 28 weeks after AOM injection in Study B, no tumors were identified either in the colon or in the small intestine in both WT and KO mice. Pathologic studies only showed more aberrant crypt with hyperchromatism and nuclear crowding in the KO than in the WT mice (Fig. 1E and F).

Study C and D: increased tumorigenesis by combination of AOM and DSS treatment

Because of the failure to induce colonic tumors by AOM alone in Study B, animals were treated with a combination of AOM and DSS by a short-term conventional method (Study C) and a long-term enhanced method (Study D).

In Study C, the gain in body weight was halted in KO but not in the WT mice (Fig. 2A). The length of the colon upon examination was 13% shorter (P < 0.05) in KO than in WT mice (Fig. 2B). The tumors were clearly identified in the distal part of the colon. Most of them were round with smooth surface. The number of tumors per mouse in KO mice was 3.5 times that in WT mice (Fig. 2C). The mean size of tumors in term of tumor surface was slightly higher in KO mice without statistical significance (Fig. 2D). During DSS treatment, 3 KO mice were dying due to severe diarrhea and dehydration. These mice were terminated and excluded from the study due to the ethical considerations. All WT mice survived.

In Study D with a long term enhanced induction of tumors, the gain in body weight was lower in the KO group compared with the
Increased β-catenin expression and nucleus translocation in both tumor and surrounding nontumor tissues of the mice

The expression and cellular localization of β-catenin is shown in Fig. 6. β-Catenin was localized peripherally in the enterocytes in WT mice. Its expression was mildly increased in the cytosol in the nontreated KO mice (Fig. 6A–C vs. D–F). After AOM/DSS treatment, in the nontumor tissues, more obviously increased expression of β-catenin throughout the entire cytoplasm was identified in the KO mice compared with the WT mice (Fig. 6G–I). In the tumors, both WT and KO mice displayed increased translocation of β-catenin to the entire cytoplasm as well as into the nucleus (Fig. 6M–R). The nuclear redistribution and staining intensity were more pronounced in tumor tissues of the KO mice as compared with the WT mice (Fig. 6Q vs. R).

Increased S1P, reduced ceramide formation in the mucosa, and increased PAF in the feces of the KO mice

To get insight into the underlying biochemical changes, we determined the levels of sphingomyelin, ceramide, and S1P in the mucosa of 6 WT and 6 KO nontreated mice. As shown in Fig. 7, the sphingomyelin levels in small intestinal and colonic mucosa did not differ (Fig. 7A); a significant decrease in ceramide (Fig. 7B) was identified in small intestine but not in colon in KO mice. Interestingly, S1P levels (Fig. 7C) in the KO mice were significantly increased in both small intestine and colon, the increase being greater in colon than in the small intestine (53% vs. 30%). PAF levels in colonic mucosa (Fig. 7D) and feces (Fig. 7E) from 10 WT and 7 KO mice after treatment with AOM/DSS in Study C were measured. The results revealed more than 200% increase of PAF in the feces of KO mice (P < 0.01) compared with that in the WT mice, whereas no significant changes in colonic mucosa were identified.

Discussion

That intestinal alk-SMase may protect colon from tumorigenesis has been suggested for about two decades based on indirect
Lack of NPP7 Enhances Colonic Tumorigenesis

Figure 7.
Changes of sphingomyelin, ceramide, S1P, and PAF in the WT and KO mice. The mucosa of small intestine and colon in nontreated WT and KO mice was scraped and lysed. The sphingolipids in the lysates were extracted and subjected to mass spectrometry analysis and adjusted with the protein levels in the samples. The levels of sphingomyelin, ceramide, and S1P are shown in A to C, respectively. For PAF analysis, the colonic mucosa from the AOM/DSS-treated mice was scraped and lysed as above. The fecal samples were collected, dried, and suspended in saline. The PAF levels in the lysate and supernatant of fecal suspensions were determined by ELISA. The values were adjusted with protein levels in the lysate or dried fecal weight. Changes of PAF in mucosa (D) and in feces (E); *, P < 0.05 and **, P < 0.01, compared with the WT mice.

The tumorigenesis was positively correlated with the strength of carcinogenic treatment. Although the conventional method with one exposure to AOM and three cycles of DSS increased number and incidence but not size of the tumors in KO mice, the enhanced method with three exposures of AOM and three cycles of DSS increased all incidence, number, and size of the tumors. The 3 KO mice sacrificed in Study C were due to severe diarrhea and dehydration after DSS treatment, indicating that the young KO mice are more sensitive to DSS treatment compared with WT mice. Furthermore, all tumors induced in the WT mice were characterized as adenomas, whereas about one third of the tumors in KO mice were adenocarcinomas, indicating that lack of alk-SMase facilitates both tumorigenesis and malignant transformation of tumorigenesis in the colon.

The cancer-preventing effect of alk-SMase is not manifested until the organism encounters carcinogenic factors, because no spontaneous tumors were identified up to the age of 17 months in the KO mice. Without exposure to carcinogen, the phenotype of the KO mice is largely normal, although the mucosa of both colon and small intestine show signs of hypertrophy. This sign was reported before (10) and confirmed in the present study. The hypertrophy may be linked to the increased S1P and β-catenin as found in the nontreated KO mice. These changes are themselves probably benign and may not lead to spontaneous tumorigenesis. Upon exposure to carcinogens, the risk of dysplasia increased in alk-SMase KO mice, as shown by the increased formation of ACF, an early marker of dysplasia (23, 28). However, the ACF formed did not eventually result in visible tumorigenesis after exposure of AOM. The failure may be related to the genetic predisposition of the mice. Previous studies have shown that C57BL/6J mice are refractory to AOM, and a combination of AOM and DSS is required to induce colon cancer in these mice (29, 30). The genetic background of alk-SMase KO mice is C57BL/6, which is a founder line of C57BL/6J.

Using the combined treatment of AOM and DSS, we induced colonic tumors in both WT and KO mice. Both the incidence and tumor number were significantly higher in KO than in WT mice. The tumorigenesis was positively correlated with the strength of carcinogenic treatment. Although the conventional method with one exposure to AOM and three cycles of DSS increased number and incidence but not size of the tumors in KO mice, the enhanced method with three exposures of AOM and three cycles of DSS increased all incidence, number, and size of the tumors. The 3 KO mice sacrificed in Study C were due to severe diarrhea and dehydration after DSS treatment, indicating that the young KO mice are more sensitive to DSS treatment compared with WT mice. Furthermore, all tumors induced in the WT mice were characterized as adenomas, whereas about one third of the tumors in KO mice were adenocarcinomas, indicating that lack of alk-SMase facilitates both tumorigenesis and malignant transformation. The results are reminiscent of our previous findings in humans that malignant transformation is associated with a progressive reduction of alk-SMase from 25% in chronic colitis to 75% in colon cancer (16–18).

Biochemical analysis found several changes that provide insight of the mechanism related to the enhanced susceptibility of carcinogenesis in the KO mice. Significant reduction of ceramide was found in small intestinal mucosa not the colon. This is in agreement with the fact that alk-SMase level is high in the mucosa of small intestine and low in colon (16). However, it is worthwhile noting that the ceramide generation from diet was sharply decreased both in small intestinal content and feces by up to 90% in the KO mice (10). The second finding related to the mechanism is the increased PAF level in the intestinal lumen after AOM/DSS treatment. PAF is a proinflammatory and proliferative factor that can be synthesized rapidly in inflammatory tissues and released into the extracellular environment to trigger biologic effects via specific receptors on the cell membrane (31–33). Increased PAF levels have been found in ulcerative colitis, necrotizing enterocolitis, and colorectal cancer (31, 34). Alk-SMase, by cleaving phosphocholine from PAF, induces inactivation of...
PAF (15), thereby evoking anti-inflammatory and anticancer effects. Of interest, but not expected is the finding of increased S1P in the KO mice in both small intestine and colon with the increase in colon predominant. Among sphingolipid metabolites, S1P is a key molecule with cancer-promoting effects (35). Inhibitors of SpHk suppressed pathogenesis of colorectal tumors and colitis (36). The question how lack of alk-SMase results in an elevated S1P level cannot be simply answered because of the complexity of sphingomyelin metabolism pathways. The S1P levels are affected by SpHk to generate S1P, S1P phosphatase and S1P lyase to eliminate S1P, ceramidase to provide sphingosine for S1P formation, and also NPP2 and NPP6, which hydrolyze sphingosyl-phosphocholine to generate S1P and sphingosine, respectively (37). To reveal the biologic mechanism leading to elevated S1P requires detailed lipidomic and proteomic analysis in future studies.

The increased S1P and PAF levels in the KO mice explain not only the tumorigenesis but also the increased expression and nuclear translocation of β-catenin, a key transcriptional factor for cell survival (38), because both S1P and PAF are known to stimulate synthesis and translocation of β-catenin in cancer cells, including intestinal epithelial cells (39–41). Overexpression of PAF acetylhydrolase reduces the amount of nuclear β-catenin and induces a shift of the protein from the nucleus to the cytoplasm (42). It is interesting that an increased β-catenin is already demonstrated in nontreated KO mice, which may be responsible for the hypertrophy of the gut and also render the animal at a predisposition in response to carcinogens.

Finally our results could have implications in cancer prevention and treatment. As we showed before, tumorigenesis is associated with a progressive reduction of alk-SMase activity, and the activity can be increased by factors known to counteract colon cancer such as soluble fiber, 5-ASA, and ursodeoxycholic acid, and decreased by AOM and high-fat diet supposed to enhance colon cancer development (16, 43). Providing recombinant alk-SMase may reduce susceptibility to colon cancer induced by carcinogens, and benefit patients with high risk of colon cancer. At least in animal studies installment of recombinant alk-SMase has been shown to improve the colitis induced by DSS (44).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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