

Purification, localization, and expression of human intestinal alkaline sphingomyelinase

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Abstract Sphingomyelin (SM) metabolism in the gut may have an impact on colon cancer development. In this study, we purified alkaline sphingomyelinase (alk-SMase) from human intestinal content, and studied its location in the mucosa, expression in colon cancer, and function on colon cancer cells. The enzyme was purified by a series of chromatographies. The molecular mass of the enzyme is 60 kDa, optimal pH is 8.5, and isoelectric point is 6.6. Under optimal conditions, 1 mg of the enzyme hydrolyzed 11 mM SM per hour. The properties of the enzyme are similar to those of rat intestinal alk-SMase but not to those of bacterial neutral SMase. Immunogold electronmicroscopy identified the enzyme on the microvillar membrane in endosome-like structures and in the Golgi complexes of human enterocytes. The expression and the activity of the enzyme were decreased in parallel in human colon cancer tissues compared with the adjacent normal tissue. The enzyme inhibited DNA biosynthesis and cell proliferation dose dependently and caused a reduction of SM in HT29 cells. **Intestinal alk-SMase is localized in the enterocytes, down-regulated in human colon cancer, and may have antiproliferative effects on colon cancer cells.**—Duan, R-D., Y. Cheng, G. Hansen, E. Hertervig, J.-J. Liu, I. Syk, H. Sjöström, and Å. Nilsson. **Purification, localization, and expression of human intestinal alkaline sphingomyelinase.** *J. Lipid Res.* 2003. 44: 1241–1250.

Supplementary key words colon cancer • apoptosis • proliferation • sphingolipid

Metabolism of sphingomyelin (SM) generates the lipid messengers ceramide, sphingosine, and sphingosine-1-phosphate, which may have antiproliferative and apoptotic effects in many cell types (1–3). Several observations suggest a relationship between SM metabolism and colon cancer development (4). When rats were treated with a chemical carcinogen that could induce colon cancer, an

accumulation of SM in the colonic mucosa occurred before the onset of tumorigenesis (5). Supplementing SM and other derivatives of ceramide in the diet inhibited the formation of aberrant crypt foci, i.e., the earliest sign of tumor development, in mice treated with 1,2-dimethylhydrazine (6–8). In cultures of human colon cancer cells, ceramide and sphingosine were found to arrest the cell cycle at G2/M phase and caused accumulation of cells in the S phase (9). It was recently reported that ceramide and sphingosine reduced the levels of cytosolic and nuclear β -catenin, indicating an effect on the APC/ β -catenin system (10). These findings link the effects of sphingolipids in the colon to a major signaling pathway responsible for the initial tumor formation in colon.

Three decades ago, Nilsson identified a sphingomyelinase (SMase) activity with optimum alkaline pH in the intestinal tract of rats, pigs, and humans (11). Differing from other types of SMase, such as acid and neutral SMase, which have been purified and cloned (12, 13), the alkaline SMase (alk-SMase) activity was only found in the intestinal tract of many species and additionally in human bile (14–16). The enzyme was present in human meconium, in the intestine of germ-free mice, and in sterile human bile, indicating that it did not originate from intestinal bacteria (11, 14). We recently purified and characterized rat intestinal alk-SMase and showed that the enzyme was only expressed in the intestine and not in other organs in rats (16). Studies of the longitudinal distribution in the gut demonstrated the highest activity of the enzyme in the jejunum and ileum, where most of the digestion of dietary SM to ceramide occurs (17).

We previously found a significant reduction of the enzyme activity in human colorectal adenocarcinomas and in the mucosa of patients with familial adenomatous polyposis (FAP) (18, 19). In addition, in patients with long-

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standing extensive ulcerative colitis that is associated with an increased risk of colon cancer, alk-SMase activity was decreased (20). Furthermore, ursodeoxycholate, a bile salt known to have anticarcinogenic effects in colon, was found to increase the colonic alk-SMase activity (21). Thus, several pieces of evidence suggest that metabolites generated by alk-SMase may influence cellular growth and differentiation in the gut.

In addition, SM metabolism in the gut may affect intestinal lipoprotein metabolism and sterol absorption. It has been demonstrated that hydrolysis of membrane SM in cultured intestinal cells influenced both sterol absorption (22, 23) and lipoprotein secretion (24), and that ceramide hydrolysis could affect the size and composition of lipoproteins secreted (25). In the gut lumen, the physical interaction between sterols and SM influences the course of both sterol absorption and SM hydrolysis (26, 27)

In the present study, we purified human intestinal alk-SMase and located the enzyme in the intestinal mucosa using a polyclonal antiserum. The expressions of the enzyme in normal and colonic cancer tissue were compared and the potential effects of the enzyme on proliferation and apoptosis of human colon cancer cells were studied.

MATERIALS AND METHODS

Materials

Human small intestinal contents were collected from ileostomy stomas of six individuals who had undergone a colectomy due to ulcerative colitis more than 3 months earlier. Before sample collection, the patients had been fasted overnight. Samples from human colonic tumors and surrounding normal tissues were obtained from patients undergoing resection at the Department of Surgery, Malmö University Hospital. Human colonic biopsy samples were taken during colonoscopic examinations at the Gastroenterology Division, Lund University Hospital. All studies involving humans had been approved by the Human Ethics Committee, Lund University.

Bovine milk SM was provided by Dr. Lena Nyberg at Skane Dairy Co. (Malmö, Sweden) and labeled with [N - ^{14}C - CH_3]choline by the methods of Stoffel (28). The specific activity of [^{14}C]SM was 56 μ Ci/mg. DEAE Sepharose, Sephadex G25 (PD10) column, and Phenyl Sepharose 6 FF were obtained from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Uno Q anion exchange chromatography cartridge, prepacked SE gel chromatography column, Biologic HR protein purification system, and isoelectrofocusing instrument Rotofor were obtained from Bio-Rad (Sundberg, Sweden). YM 30 filtration membranes with 30 kDa molecular mass cut-off were purchased from Amicon (Beverly, MA). HT-29 cells were obtained from American Tissue Culture Collection (Rockville, MD). The cell death detection kit and the cell proliferation reagent 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]1,3-benzene disulfonate (WST-1) were purchased from Roche Diagnostics GmbH (Mannheim, Germany). [3H]thymidine and [3H]choline chloride were bought from DuPont Corporation (Cambridge, MA).

Purification procedure

The human intestinal contents were freeze dried and dissolved in ice-cold 0.15 M NaCl containing 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride (PMSF). The nonsolu-

ble materials were removed by centrifugation at 8,000 rpm for 15 min at 4°C. The proteins were precipitated by acetone as previously described (16) and dissolved in 20 mM Tris-HCl buffer, pH 8.2, containing 0.075 M NaCl, 1 mM benzamidine, and 1 mM PMSF (DEAE buffer). The sample was subjected to a series of chromatographies by a means very similar to that used for purification of rat alk-SMase (16). Briefly, the sample was first loaded on a DEAE Sepharose column that had been equilibrated with DEAE buffer. After loading, the column was eluted with the same buffer and the fractions containing nonretained proteins were collected. The column was then eluted with the same buffer but contained 0.5 M NaCl to obtain the retained proteins. The intestinal alk-SMase activity was in the fractions containing nonretained proteins. These fractions were pooled, supplemented with ammonium sulfate to 1.0 M, and applied to a column packed with Phenyl Sepharose for hydrophobic interaction chromatography (HIC). The column was eluted with a gradient of ammonium sulfate from 1.0 to 0 M in 20 mM Tris-Maleate buffer (pH 7.0). After HIC, the fractions containing alk-SMase activity were pooled and desalted, followed by loading on a Uno Q column for high-affinity anion exchange chromatography. The column was eluted with a gradient of NaCl from 0 to 0.25 M in 20 mM Tris buffer (pH 7.0). The fractions with high enzyme activity were pooled, concentrated by ultra filtration, and loaded on an SE column, followed by elution with 20 mM Tris, 0.15 M NaCl (pH 8.2). The fractions with high alk-SMase activity were subjected to native isoelectric focusing electrophoresis with pH ranging from 3 to 10. The protein concentrations during chromatographies were monitored by a UV detector or quantified by a kit from Bio Rad with bovine albumin as a standard. The purity of the enzyme was visualized by 10% SDS polyacrylamide gel electrophoresis (PAGE) and the gel was stained by silver staining.

SMase assay

Alk-SMase activity was determined by two methods according to Duan and Nilsson (29). For monitoring the migration of the activity during the purification procedure, 2 μ l of samples from fractions collected were added to the tubes followed by adding 50 mM Tris-HCl buffer (pH 9.0) containing 0.15 M NaCl, 2 mM EDTA, 10 mM taurocholate (TC), 0.1 mM SM, and 0.80 μ M [^{14}C]SM (\sim 8,000 dpm) to a final volume of 100 μ l. After incubation at 37°C for 30 min, the reaction was terminated by adding 0.4 ml of chloroform-methanol (2:1, v/v) followed by centrifugation at 10,000 rpm for 10 s. An aliquot (100 μ l) of the upper phase containing the cleaved phosphocholine was analyzed for radioactivity by liquid scintillation. The activity was expressed as dpm in the upper phase after 30 min incubation. For assaying the enzyme activity in HT 29 cells, tissue samples, and in characterization studies, 5 μ l of samples were added in 50 mM Tris-HCl buffer containing 0.15 M NaCl, 2 mM EDTA, and 10 mM TC (pH 9.0) (assay buffer) to a final volume of 80 μ l. The reaction was started by adding 80 pmol [^{14}C]SM (\sim 8,000 dpm) in a 20 μ l assay buffer and incubating at 37°C for 30 min. The reaction was terminated as above, and the activity was calculated and expressed as nmol substrate hydrolyzed by 1 mg sample protein in 1 h (nmol/h/mg).

The hydrolytic capacity of the alk-SMase was determined by adding 5 ng enzyme in 100 μ l assay buffer containing different amounts of SM ranging from 5 μ g to 640 μ g together with 100 pmol of [^{14}C]SM. Percentage of hydrolysis of the substrate was calculated from the ratio of dpm in the upper phase to the total dpm added in the system. The mass of SM that had been hydrolyzed in 1 h was calculated from the percentage hydrolysis. The Lineweaver-Burk plot was used to determine the V_{max} of the enzymatic reaction under the assay condition.

Antibody preparation

The purified alk-SMase was subjected to 10% SDS-PAGE and stained by 0.1% Coomassie blue. The enzyme band was excised and homogenized and the homogenates were used to immunize two rabbits once a month for 3 months by subcutaneous injections. Bleeding started 2 weeks after the second injection. The immune procedure was performed by the company Agri Sera AB (Vännäs, Sweden).

Immunogold electron microscopy

Human normal ileum biopsy samples were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h at 4°C. After a rinse with 0.1 M phosphate buffer (pH 7.2), the small intestinal samples were infused with 2.3 M sucrose for 30 min and then mounted on top of a metal pin and frozen in liquid nitrogen. Ultracryosections (~50 nm) were cut in an RMC 6,000 XL ultracryomicrotome and collected with a sucrose droplet and attached to formvar and carbon-coated nickel grid. Immunogold labeling was performed according to Hansen et al. (30) using either the rabbit anti-human SMase serum or the preimmune serum as a control. The ultracryosections were finally examined in a Zeiss EM900 electron microscope and electronmicrographs were obtained using a Mega View II CCD camera and an Image analysis system.

Western blot

The tissue samples were homogenized in 50 mM Tris buffer containing 2 mM EDTA, 10 mM TC, 1 mM PMSE, 1 mM benzamide, and 0.5 mM DTT (pH 7.4), followed by sonication for 10 s. After centrifugation at 13,000 rpm for 10 min at 4°C, 50 µg proteins of each sample were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane by electrophoresis. The membrane was blocked in 20 mM Tris buffer containing 0.15 M NaCl and 5% fat free dried milk for 2 h and probed with antiserum (1:500) for 2 h. After washing, the membrane was incubated with biotinylated goat anti-rabbit antibody for 2 h and then with a complex of equal amounts of streptavidin and biotinylated-alkaline phosphatase for 3 h. After color development, the density of the protein bands was quantified by Scion Image software program.

Effects of alk-SMase on cell proliferation and apoptosis in HT29 cells

HT29 cells were cultured in RPMI-1640 medium with L-glutamine containing 100 IU/ml penicillin, 10 µg/ml streptomycin, and 10% (v/v) heat inactivated fetal calf serum. The purified alk-SMase was diluted to a concentration with an activity being 200 nmol/h/ml. HT29 cells seeded in 96 well plate were incubated with the alk-SMase in different doses for 24 h. WST-I reagent was added in each well and cell proliferation was determined as previously described (31). For assaying DNA replication, the cells were treated with alk-SMase for 24 h. [³H]thymidine at a dose of 0.5 µCi/well was added and the incorporation of [³H]thymidine in DNA was determined as described (31).

Apoptosis was determined by ELISA using a cell death detection ELISA kit, which is based on the quantification of mono- and oligonucleosomes in the cytoplasm. The cells were treated with alk-SMase for 24 h and lysed by the lysis buffer provided with the kit. The cytoplasmic histone-associated DNA fragments were quantified, and the specific enrichment was determined as described previously (31).

Measurement of SM in the cells

Determination of cellular SM levels was performed according to Hedlund et al. (32). In brief, the cells were preincubated with [³H]choline for 48 h at a concentration of 0.5 µCi/ml and then

treated with alk-SMase as described above. The total lipids were extracted according to Bligh and Dyer (33) and applied on Silica gel plate (60 F, 0.25 mm) for thin layer chromatography. The plates were developed by chloroform-methanol-25% ammonium hydroxide (65:25:4, v/v/v) and the lipid bands were visualized by iodine vapor. The SM bands were scraped according to the internal standard and the radioactivity in bands was measured by liquid scintillation counting.

RESULTS

Purification

In the initial chromatographic separation on DEAE-Sephacrose column, two portions with alk-SMase activity were identified (Fig. 1). The activity in the first portion (Portion A) was not retained on the column and eluted with the loading buffer (20 mM Tris buffer pH 8.2 with 0.075 M NaCl). A second and smaller peak of alk-SMase (Portion B) was eluted by increasing NaCl concentration to 0.5 M in the same buffer. According to our previous studies, portion A contains intestinal alk-SMase, whereas portion B is likely represent bile alk-SMase (16, 34). Portion A was saved and subjected to Phenyl Sepharose HIC (Fig. 2A), Uno Q high affinity anion chromatography (Fig. 2B), and SE chromatography (Fig. 2C). The fractions containing alk-SMase were marked by arrows in each panel. The alk-SMase activity in SE gel chromatography was found in fraction 28 to 33 with peak activity in fraction 30 and 31. The proteins in these fractions were visualized by 10% SDS-PAGE (Fig. 3A). A band at about 60 kDa (marked by arrow) was identified as correlated with the enzyme activity. To further confirm that this 60 kDa protein is alk-SMase, fractions 30 and 31 were combined and subjected to native electric focusing electrophoresis. A total of 20 fractions were collected. The highest alk-SMase activity was found in the fraction with pH 6.6. SDS-PAGE confirmed that the fraction contained a single protein with a mass of 60 kDa (Fig. 3B), which was identical to those shown in Fig. 3A.

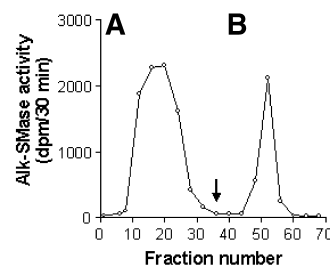
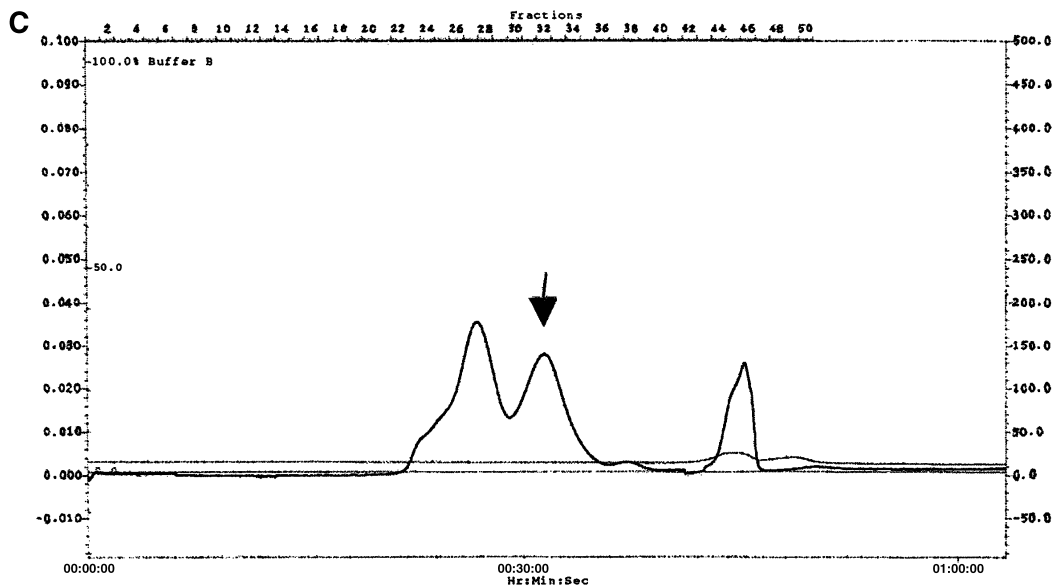
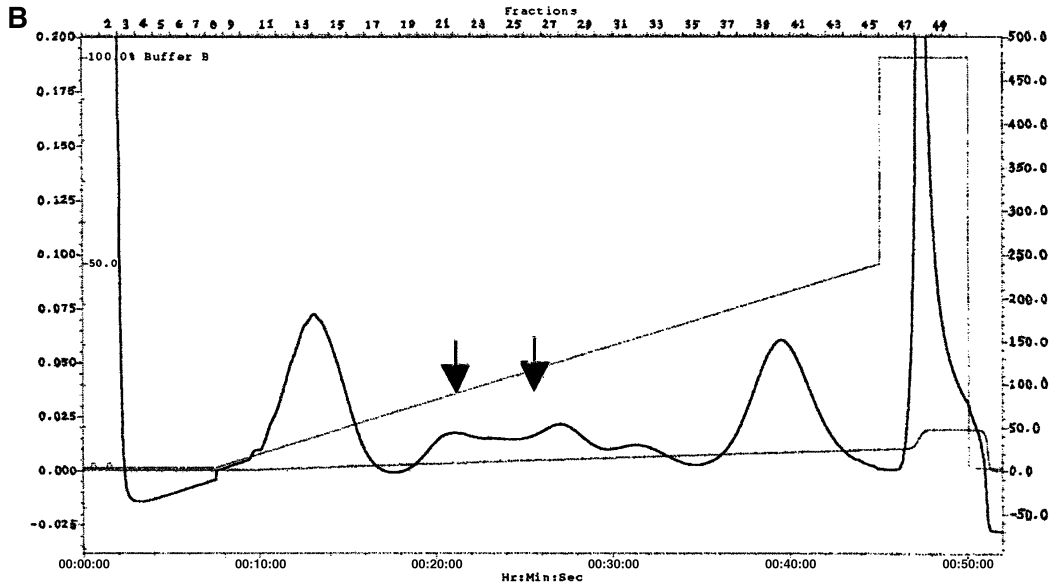
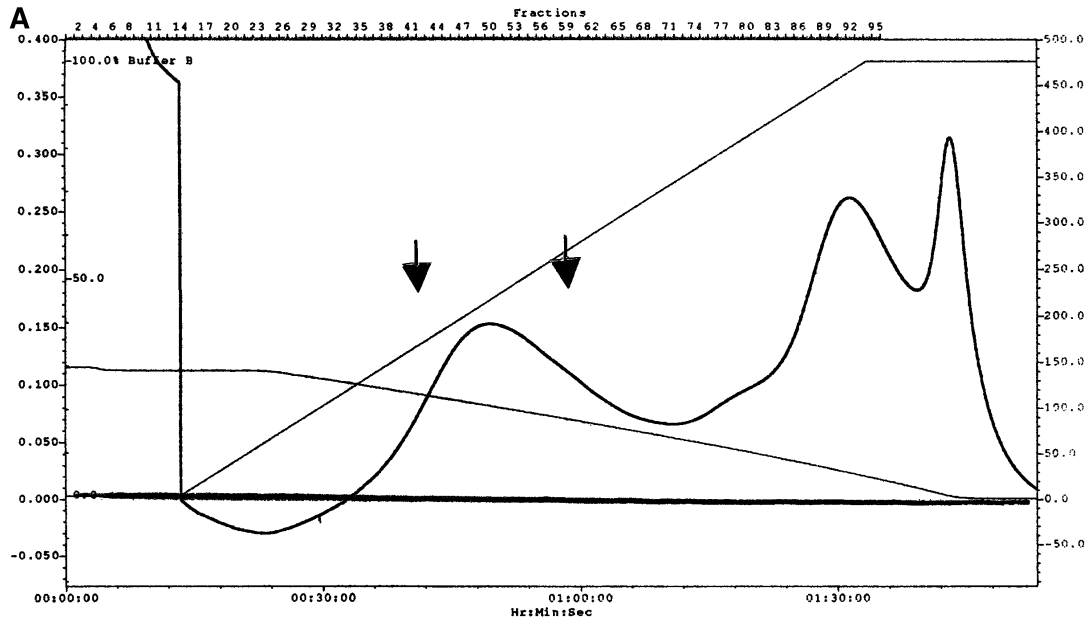


Fig. 1. Profile of DEAE sepharose chromatography. Proteins dissolved in 20 mM Tris buffer containing 0.075 M NaCl, 1 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride were loaded on a DEAE column, followed by washing the column with the same loading buffer. The column was then eluted with the buffer containing 0.5 M NaCl, as indicated by the arrow. Alkaline sphingomyelinase (Alk-SMase) activity in the fractions was determined. Portion A contains the activity that was not retained on the column and portion B, the enzyme retained.



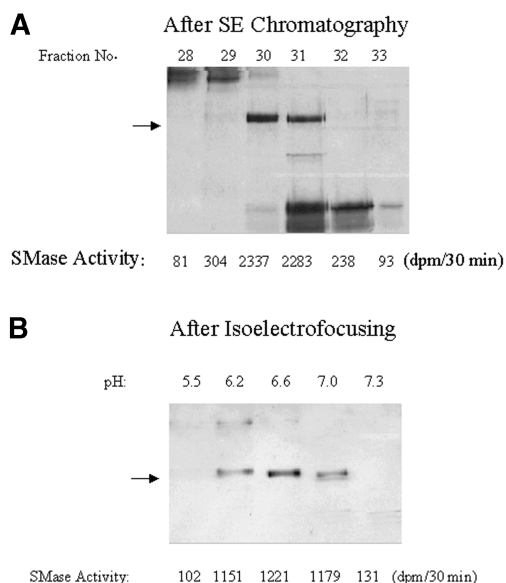


Fig. 3. SDS-PAGE. A: Twenty microliters from each fraction after SE chromatography was subjected to 10% SDS-PAGE and the gel was stained by silver staining. The fraction numbers are shown above the panel and SMase activities below it. The bands indicated by an arrow are the ones correlated with SMase activity. B: Fraction 30 and 31 from SE chromatography were combined and subjected to electrofocusing. The fractions with alk-SMase activity after electrofocusing were resolved by 10% SDS-PAGE and the gel was stained by silver staining. The pH values of each fraction are shown at the top of the panel and SMase activity at the bottom. The bands indicated by an arrow are purified alk-SMase.

Characteristics of human alk-SMase

The optimal pH, divalent ion dependency, bile salt dependency, glutathione inhibition, and substrate specificity of alk-SMase were examined according to methods described previously (16). The pH dependence was similar to that of rat intestinal alk-SMase. Maximal activity was obtained at pH 8.5 and the activity at pH 7.5 was about 68% of the maximal activity. Like the rat enzyme, human alk-SMase did not require Ca^{2+} and Mg^{2+} and was not inhibited by EDTA. In addition, the activity at alkaline pH without divalent cations remained significantly higher than that at pH 7.5 with Mg^{2+} present. In analogy with rat alk-SMase, the enzyme activity was specifically stimulated by TC and taurochenodeoxycholate, but inhibited by other detergents such as CHAPS and Triton X-100. Glutathione, which inhibits neutral SMase, had no significant effects on human alk-SMase. Although the major product generated by the enzyme was ceramide, the enzyme had weak activity against phosphatidylcholine at neutral pH in the presence of Ca^{2+} . Because these properties described above are very similar to those for rat intestinal alk-SMase reported

TABLE 1. Comparison of properties of intestinal alkaline SMases in rat and human

	Rat	Human
Molecular mass (kDa)	58	60
Optimal pH	9.0	8.5
Isoelectric point	6.2	6.6
V_{max} (mmol/h/mg)	0.93	11.0
Stimulation by bile salt	TC, TCDC	TC, TCDC
Glutathione inhibition	no	no
Mg^{2+} dependence	no	no
Effect of EDTA	no	no
Cross immunoreaction	yes	yes
Activity against PC	yes	yes

PC, phosphatidylcholine; SMase, sphingomyelinase; TC, taurocholate; TCDC, taurochenodeoxycholate. The comparison was made based on the results in this paper and those in our previous investigation (16). The pI value in rat alkaline SMase is from our unpublished data.

previously (16), detailed data are not presented, but the properties for rat and human alk-SMase are summarized in **Table 1**.

To determine the hydrolytic capacity of the enzyme, the activity of alk-SMase (5 ng) was assayed in the presence of different amounts of SM in 100 μl assay buffer (**Fig. 4A**). The V_{max} was determined by a Lineweaver-Burk plot (**Fig. 4B**). One milligram of the enzyme is able to hydrolyze about 11 mmol SM in 1 h.

Localization of alk-SMase in human small intestine

By immunogold labeling, alk-SMase was found to be localized at the surface of the microvillar membrane in the enterocytes (**Fig. 5A**). Specific labeling of SMase was also identified in endosome-like structures situated beneath the microvillar membrane (**Fig. 5C**) and in Golgi complexes (unpublished observations). No specific labeling of alk-SMase was found in the enterocytes using the preimmune serum as controls (**Fig. 5B, D**).

Cross reaction of the rabbit anti-human alk-SMase antiserum

Figure 6 shows the cross reaction of rabbit anti-human alk-SMase with the enzyme in rat intestine and human bile. In human bile, the antibody identified a band with similar molecular mass as that of human intestinal alk-SMase, and in rat intestinal tissue, a band that was slightly smaller (58 kDa) than that of human alk-SMase. The molecular mass of the band identified in rat intestine is equal to that of purified rat intestinal alk-SMase (16). However, no positive band was identified when the antiserum was used against bacterial neutral SMase, as shown in **Fig. 6D** compared with **Fig. 6E**. The negative result with neutral SMase was not due to a low amount of protein loaded, as silver staining clearly showed a major band and two small bands in the neutral SMase preparations (**Fig. 6F**).

Fig. 2. Profiles of chromatographies. The portion A from DEAE chromatography was subjected to Phenyl Sepharose hydrophobic interaction chromatography (HIC) (A), Uno Q anion exchange chromatography (B), and SE gel chromatography (C). Ammonium sulfate gradient in HIC was from 1.0 to 0 M, and NaCl gradient in anion exchange chromatography was from 0 to 0.25 M. Protein concentrations were monitored by UV detector. The alk-SMase activities were determined and the fractions with high activity are indicated by the arrows.

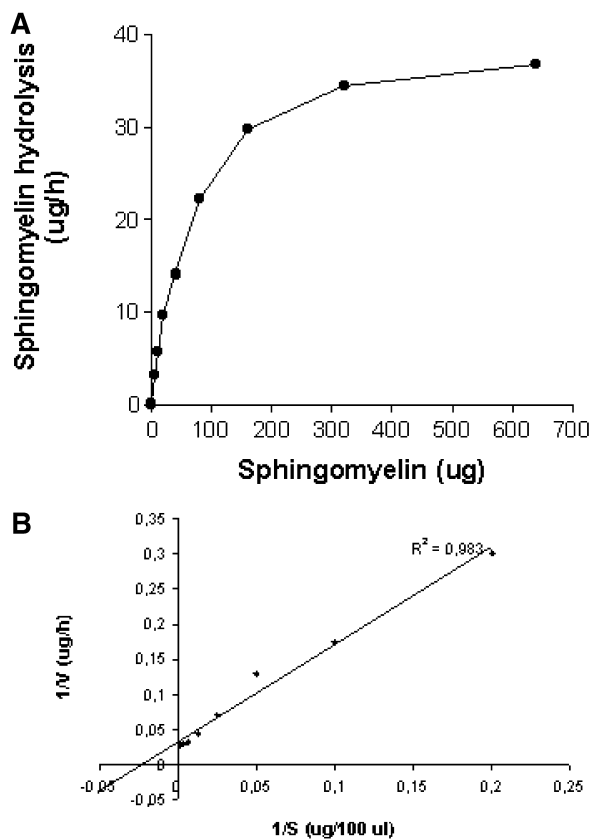


Fig. 4. The hydrolytic capacity of purified alk-SMase. The enzyme activity was determined in the assay buffer in the presence of various concentrations of unlabeled sphingomyelin (SM), and 0.1 μ mole [14 C]SM. A: Shows the substrate saturation curve and B is the Lineweaver-Burk plot. Results shown are representative of three determinations.

Comparing the expression of alk-SMase in normal and colon cancer tissues

Previous studies have identified a reduction of alk-SMase activity in human carcinomas and colonic adenomas (18, 19). To extend these previous findings, Western blot was performed to examine the enzyme levels in human colon cancer tissues in comparison with the adjacent normal tissues in six individuals (Fig. 7). In pairwise comparisons, the levels of the enzyme were higher in the normal tissues than in the cancers. The immunoreactivity data and enzyme activity data are summarized in Fig. 8. The mean densities of the enzyme bands were decreased by 39% ($P < 0.05$) and the maximal density reduced by 51% in the tumors. The reduction in immunoreactivity was largely in agreement with the changes in enzyme activities in each individual case. However, as shown in Fig. 7, the variations among individuals were big.

Effects of alk-SMase on proliferation and apoptosis of HT29 cells

To examine the potential anticancer effect of alk-SMase on human colon cancer, HT29 cells were incubated with the purified enzyme. As shown in Fig. 9A, when HT29 cells were treated with the enzyme for 24 h, the cell prolifer-

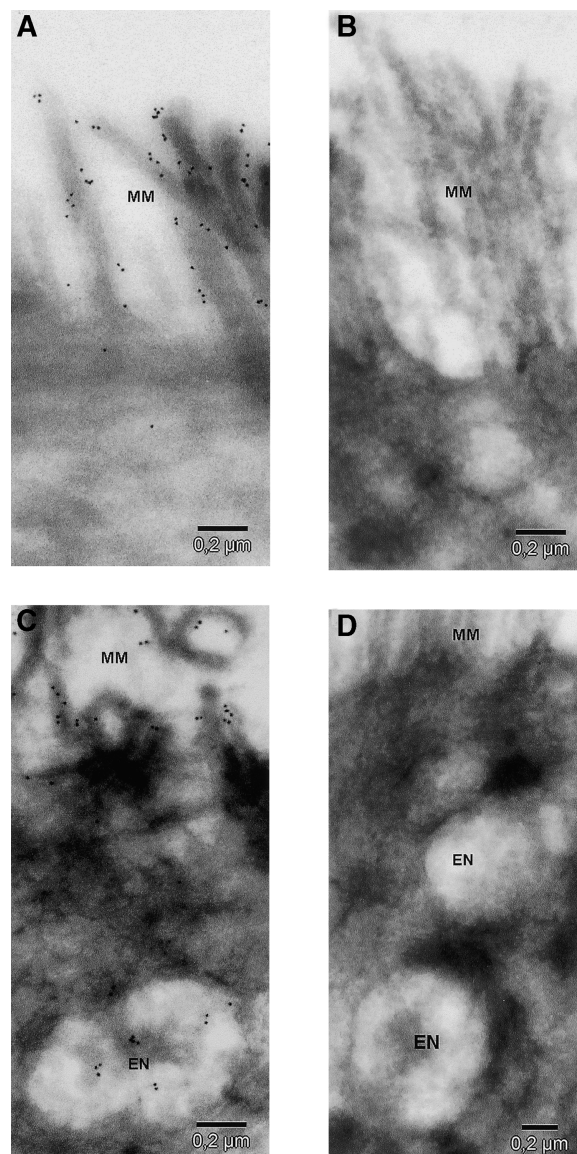


Fig. 5. Indirect immunogold labeling of human small intestinal enterocytes using the polyclonal rabbit anti-human alk-SMase (A and C), or the corresponding preimmune serum (B and D). Immunogold labeled particles of alk-SMase were observed in the microvillar membrane (MM) and in endosome-like structure (EN). Bars indicate 0.2 μ m.

eration rates were decreased, and the incorporation of [3 H]thymidine was inhibited in a dose-dependent manner (Fig. 9A). At the dose that inhibited cell proliferation, the enzyme decreased SM levels in the cells by about 25% (Fig. 9B). However, the enzyme did not stimulate apoptosis in HT 29 cells in the doses tested (Fig. 9C).

DISCUSSION

In the present study, we purified human intestinal alk-SMase, and for the first time demonstrated the localization of the enzyme to the brush border of the intestinal mucosal cells. As a general feature, the enzyme is very sim-

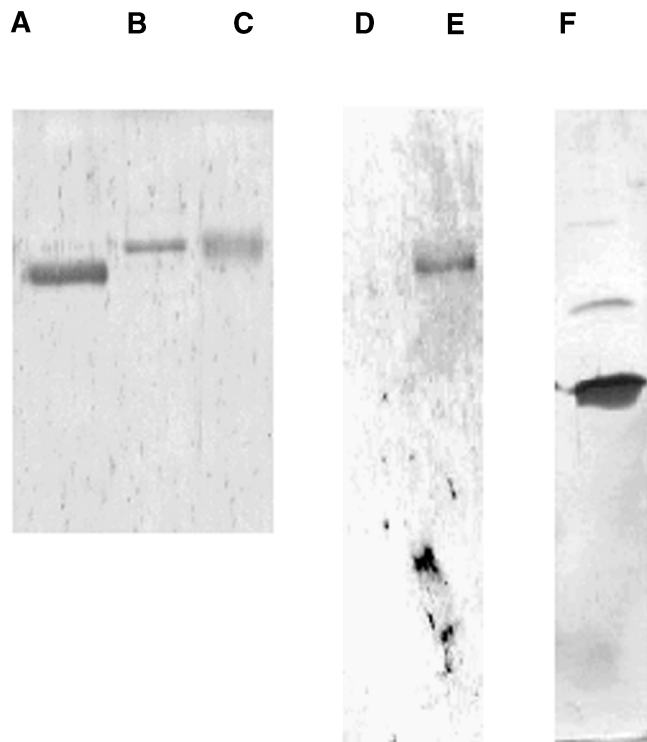
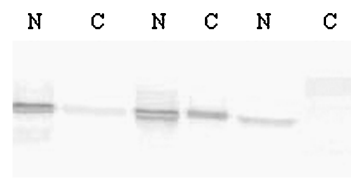


Fig. 6. Western blot showing cross reaction of rabbit anti-human alk-SMase antiserum with rat intestinal alk-SMase. A: Rat intestine; B, human bile; C, human small intestine; D, bacterial neutral SMase; E, human small intestine. F: Shows the silver staining of the sample in D.

ilar to rat intestinal alk-SMase that was purified in an earlier study, judging from the hydrolytic properties, the molecular mass, and the immunologic cross reactivity. The paper also extends our earlier studies showing decreased alk-SMase activity in colon tumors (18, 19) by demonstrating that the decreased enzyme activity is paralleled by a decrease in enzyme expression. Finally, the study shows that the purified enzyme is able to inhibit proliferation and reduce SM levels of colon carcinoma HT29 cells.

The starting material for the purification was intestinal content collected from ileostomy patients. The procedure took advantage of our earlier experience in the rat study (16, 35) that much of the enzyme could be released by luminal perfusion of the small intestine by a solution containing a moderate concentration of bile salts. In line



Alk SMase:	27.5	8.3	20.1	24.2	11.2	0.90
(nmole/h/mg)						



Alk SMase	37.8	1.3	29.6	16.7	2.1	0.6
(nmole/h/mg)						

Fig. 7. Comparison of the expression of alk-SMase in colon cancer tissues with the adjacent normal tissues of six patients. Cancer and adjacent normal tissues were homogenized and 50 μ g of proteins in each sample were subjected to Western blotting analysis. N, normal adjacent tissue; C, cancer tissue. The alk-SMase activities of corresponding samples are given under each panel.

with this expectation, the ileostomy contents were found to contain considerable alk-SMase activity. A concern was that degradation of the enzyme by pancreatic proteases might have occurred. Previous studies showed, however, that the activity of intestinal alk-SMase was extremely resistant to digestion by pancreatic trypsin and chymotrypsin (4, 36). Activity of alk-SMase has also been found in feces of rats (21) and humans (unpublished observations) and was unchanged for about 2 days even at room temperature. Due to the high stability of the enzyme, it is possible to purify the enzyme from the intestinal content collected from the ileostomies.

Previous studies have shown that only humans have alk-SMase in both the intestinal mucosa and bile (14, 15). The two forms of alk-SMases share many properties in common but differed in the behavior on DEAE chromatography (34). The binding of intestinal alk-SMase on DEAE matrix is weaker than that of bile alk-SMase and in the buffer containing 0.075 M NaCl; the intestinal alk-SMase was not retained on the column, in contrast to the bile alk-SMase.

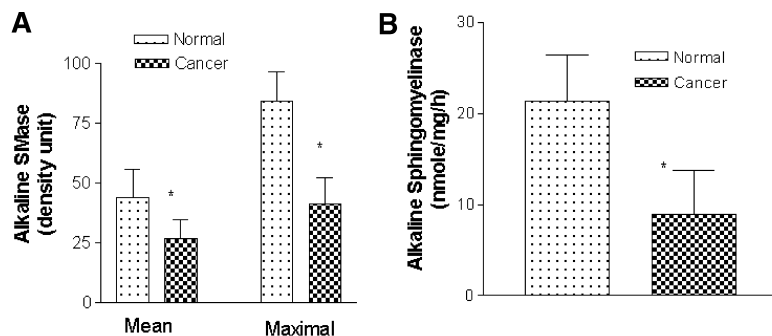


Fig. 8. Comparison of the densities of the bands (A) and alk-SMase activities (B) in normal and cancer tissues shown in Fig. 7. The mean and the maximal density of the SMase bands were measured. The alk-SMase activities were determined as described. * $P < 0.05$ compared with the normal tissues by paired Student *t*-test.

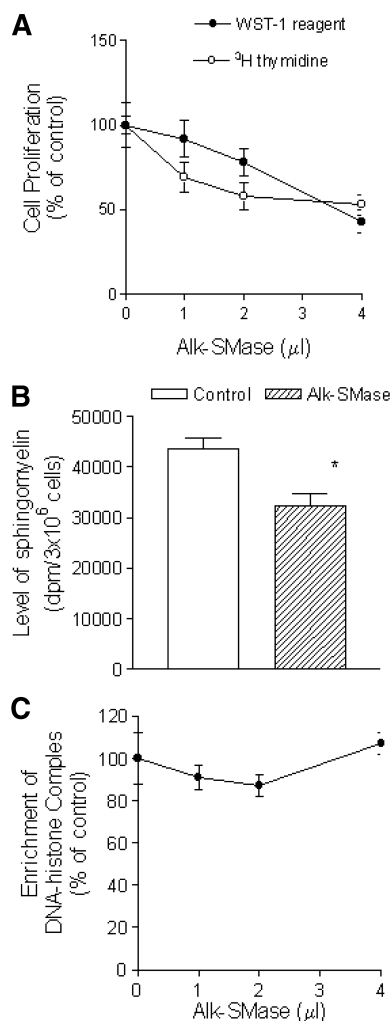



Fig. 9. Effects of human alk-SMase on cell proliferation, apoptosis, and the levels of SM in HT29 cells. The purified enzyme was diluted to activity being 200 nmol/h/ml. The cells were incubated with the enzyme at different doses for 24 h. The changes of cell proliferation rate were shown in A and apoptosis in C. B: The cells were incubated with 2 μl of alk-SMase for 24 h. The changes of SM in the cells were determined by thin layer chromatography. Results are mean ± SEM from six experiments. * $P < 0.05$ compared with control.

As with the rat alk-SMase, the human enzyme clearly differs from the bacterial neutral SMase and neutral SMase of human tissues (37). The high pH-optimum, the lack of dependence on divalent metal ions, the lack of inhibition by glutathione, the strong inhibition by certain detergents such as CHAPS and Triton X-100, and the selective stimulation by primary taurine-conjugated bile acids are typical characteristics of both rat and human alk-SMase. Furthermore, these enzymes have a similar molecular mass and exhibit immunological cross reactivity. They differ, however, in catalytic efficiency. Under the assay conditions used, the hydrolytic capacity of human alk-SMase is about 10 times greater than that of rat enzyme. Further studies elucidating the amino acid sequence and the catalytic mechanism are necessary to explain this difference.

An earlier study suggested that intestinal alk-SMase activity was enriched in the brush border of the enterocytes (11). The location of the enzyme in the gut, however, has not been clearly visualized. In this study, we demonstrated by immunogold technique that intestinal alk-SMase is present along the microvillar membrane, whereas the basolateral membrane contains little or no enzyme. This location resembles that of the enzymes catalyzing the terminal digestion of carbohydrates and proteins, e.g., lactase, aminopeptidase N, and dipeptidyl peptidase IV (38, 39). As a consequence, the enzyme can probably be released into the lumen from the intestinal mucosa by physiological concentrations of bile salts, as indicated previously (35). The enzyme was also found in endosomal-like structures situated beneath the microvillar membrane. This might indicate that *a*) the enzyme can be taken up by endocytosis, *b*) newly synthesized enzyme actually passes this compartment en route to the microvillar membrane, or *c*) a crinophagic pathway in which the enzyme is degraded.

We previously found that the activities of alk-SMase were significantly reduced in human colorectal adenomas and carcinomas and in the mucosa of FAP patients (18, 19). The finding in 18 patients in the initial study (18) was later confirmed in another 36 patients (unpublished observations). A recent study also found a reduction of the enzyme activity in human long-standing ulcerative colitis, a disease linked to an increased risk of colon cancer (20). The present work extends the previous observations by showing that the decrease in alk-SMase activity in tumors is linked to a parallel decrease in expression of the enzyme. Although the reduction of alk-SMase in the tumors compared with surrounding mucosa is a general feature, the level of alk-SMase varied considerably. At present, we do not know the genetic and external factors behind this variation, although a significant influence of dietary factors has been demonstrated in rodents (40).

To further test the hypothesis that alk-SMase may influence cell growth, its effects on human colon cancer HT29 cells were examined. The DNA replication and cell proliferation were inhibited associated with a reduction of SM in the cells. The enzyme at the dose tested did not induce apoptosis; however, an apoptotic effect of the enzyme *in vivo* cannot be excluded. A good correlation of caspase-3 activity with the activity of alk-SMase in rat was previously reported in the colon after *in vivo* administration of ursodeoxycholic acid (21), a bile acid that was found to have anticancer effects in colonocytes (41). As has been shown recently, different mechanisms might be involved in mediating the apoptotic effect of exogenous SMase (42). In addition, the cells may have different SM pools with different functions. It is possible that alk-SMase in this study only hydrolyzed SM in the outer leaflet of the membrane, which is unable to trigger apoptosis. Furthermore, the enzyme *in vivo* may be colocalized with ceramidase in the brush border to generate sphingosine that may permeate the cells to induce apoptosis, as demonstrated by Schmelz et al. (10).

In summary, we purified human intestinal alk-SMase, identified its location and down-regulation in colon tumors, and showed its potential antiproliferative action on human colon cancer cells. The further studies on the biological effects of the enzyme upon intestinal cells and its preventive role in tumorigenesis have to await the sequencing and cloning of the enzyme. 

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REFERENCES

- Hannun, Y. A., and C. M. Lincardic. 1993. Sphingolipid breakdown products: anti-proliferative and tumor-suppressor lipids. *Biochim. Biophys. Acta.* **1154**: 223–236.
- Merrill, A. H., E. M. Schmelz, E. Wang, D. L. Dillehay, L. G. Rice, F. Meredith, and R. T. Riley. 1997. Importance of sphingolipids and inhibitors of sphingolipid metabolism as components of animal diets. *J. Nutr.* **127**: 830S–833S.
- Kolesnick, R. N., and M. Kronke. 1998. Regulation of ceramide production and apoptosis. *Annu. Rev. Physiol.* **60**: 643–665.
- Duan, R-D. 1998. Hydrolysis of sphingomyelin in the gut and clinical implications in colorectal tumorigenesis and other gastrointestinal diseases. *Scand. J. Gastroenterol.* **33**: 673–683.
- Brasitus, T. A., P. K. Dudgey, and R. Dahiya. 1986. Premalignant alterations in the lipid composition and fluidity of colonic brush border membranes of rats administered 1,2 dimethylhydrazine. *J. Clin. Invest.* **77**: 831–840.
- Dillehay, D. L., S. K. Webb, E-M. Schmelz, and A. H. Merrill. 1994. Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice. *J. Nutr.* **124**: 615–620.
- Schmelz, E. M., D. L. Dillehay, S. K. Webb, A. Reiter, J. Adams, and A. H. Merrill. 1996. 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. *Cancer Res.* **56**: 4936–4941.
- Schmelz, E. M., A. S. Bushnev, D. L. Dillehay, D. C. Liotta, and A. H. Merrill. 1997. Suppression of aberrant colonic crypt foci by synthetic sphingomyelins with saturated or unsaturated sphingoid base backbones. *Nutr. Cancer.* **28**: 81–85.
- Ahn, E. H., and J. J. Schroeder. 2002. Sphingoid bases and ceramide induce apoptosis in HT-29 and HCT-116 human colon cancer cells. *Exp. Biol. Med.* **227**: 345–353.
- Schmelz, E. M., P. C. Roberts, E. M. Kustin, L. A. Lemonnier, M. C. Sullards, D. L. Dillehay, and A. H. Merrill. 2001. Modulation of intracellular beta-catenin localization and intestinal tumorigenesis in vivo and in vitro by sphingolipids. *Cancer Res.* **61**: 6723–6729.
- Nilsson, Å. 1969. The presence of sphingomyelin- and ceramide-cleaving enzymes in the small intestinal tract. *Biochim. Biophys. Acta.* **176**: 339–347.
- Quintern, L. E., E. H. Schuchman, O. Levrin, M. Suck, K. Ferlinz, H. Reinke, K. Sandhoff, and R. J. Desnick. 1989. Isolation of cDNA clones encoding human acid sphingomyelinase: occurrence of alternatively processed transcripts. *EMBO J.* **8**: 2469–2473.
- Chatterjee, S., H. Han, S. Rollins, and T. Cleveland. 1999. Molecular cloning, characterization, and expression of a novel human neutral sphingomyelinase. *J. Biol. Chem.* **274**: 37407–37412.
- Duan, R-D., E. Hertervig, L. Nyberg, T. Tauge, B. Sternby, J. Lillienau, A. Farooqi, and Å. Nilsson. 1996. Distribution of alkaline sphingomyelinase activity in human beings and animals. *Dig. Dis. Sci.* **41**: 1801–1806.
- Nyberg, L., R-D. Duan, J. Axelsson, and Å. Nilsson. 1996. Identification of an alkaline sphingomyelinase activity in human bile. *Biochim. Biophys. Acta.* **1300**: 42–48.
- Cheng, Y., Å. Nilsson, E. Tömquist, and R-D. Duan. 2002. Purification, characterization and expression of rat intestinal alkaline sphingomyelinase. *J. Lipid Res.* **43**: 316–324.
- Nyberg, L., Å. Nilsson, P. Lundgren, and R-D. Duan. 1997. Localization and capacity of sphingomyelin digestion in the rat intestinal tract. *J. Nutr. Biochem.* **8**: 112–118.
- Hertervig, E., Å. Nilsson, L. Nyberg, and R-D. Duan. 1996. Alkaline sphingomyelinase activity is decreased in human colorectal carcinoma. *Cancer.* **79**: 448–453.
- Hertervig, E., Å. Nilsson, J. Björk, R. Hultkrantz, and R-D. Duan. 1999. Familial adenomatous polyposis is associated with a marked decrease in alkaline sphingomyelinase activity: a key factor to the unrestrained cell proliferation. *Br. J. Cancer.* **81**: 232–236.
- Sjöqvist, U., E. Hertervig, Å. Nilsson, R-D. Duan, A. Ost, B. Tribukait, and R. Lofberg. 2002. Chronic colitis is associated with a reduction of mucosal alkaline sphingomyelinase activity. *Inflamm. Bowel Dis.* **8**: 258–263.
- Cheng, Y., H-T. Tauschel, Å. Nilsson, and R-D. Duan. 1999. Administration of ursodeoxycholic acid increases the activities of alkaline sphingomyelinase and caspase-3 in rat colon. *Scand. J. Gastroenterol.* **34**: 915–920.
- Chen, H., E. Born, S. N. Mathur, F. C. Johlin, and F. J. Field. 1992. Sphingomyelin content of intestinal cell membranes regulates cholesterol absorption. Evidence for pancreatic and intestinal cell sphingomyelinase activity. *Biochem. J.* **286**: 771–777.
- Field, F. J., E. Born, S. Murthy, and S. N. Mathur. 1998. Transport of cholesterol from the endoplasmic reticulum to the plasma membrane is constitutive in CaCo-2 cells and differs from the transport of plasma membrane cholesterol to the endoplasmic reticulum. *J. Lipid Res.* **39**: 333–343.
- Field, F. J., H. Chen, E. Born, B. Dixon, and S. Mathur. 1993. Release of ceramide after membrane sphingomyelin hydrolysis decreases the basolateral secretion of triacylglycerol and apolipoprotein B in cultured human intestinal cells. *J. Clin. Invest.* **92**: 2609–2619.
- Kirby, R. J., S. Zheng, P. Tso, P. N. Howles, and D. Y. Hui. 2002. Bile salt-stimulated carboxyl ester lipase influences lipoprotein assembly and secretion in intestine: a process mediated via ceramide hydrolysis. *J. Biol. Chem.* **277**: 4104–4109.
- Nyberg, L., R-D. Duan, and Å. Nilsson. 2000. A mutual inhibitory effect on absorption of sphingomyelin and cholesterol. *J. Nutr. Biochem.* **11**: 244–249.
- Eckhardt, E. R., D. Q. Wang, J. M. Donovan, and M. C. Carey. 2002. Dietary sphingomyelin suppresses intestinal cholesterol absorption by decreasing thermodynamic activity of cholesterol monomers. *Gastroenterology.* **122**: 948–956.
- Stoffel, W. 1975. Chemical synthesis of choline-labeled lecithins and sphingomyelin. *Methods Enzymol.* **36**: 533–541.
- Duan, R. D., and Å. Nilsson. 1999. Enzymes hydrolysing sphingolipids in gastrointestinal tract. *Methods Enzymol.* **311**: 276–286.
- Hansen, G. H., L. Immerdal, E. Thorsen, L-L. Niels-Christiansen, B. T. Nystrom, E. J. F. Demant, and E. M. Danielsen. 2001. Lipid rafts exist as stable cholesterol-independent microdomains in the brush border membrane of enterocytes. *J. Biol. Chem.* **276**: 32338–32344.
- Liu, J-J., Å. Nilsson, S. Oredsson, V. Badmaev, W-Z. Zhao, and R-D. Duan. 2002. Boswellic acids trigger apoptosis via a pathway dependent on caspase-8 activation but independent of Fas/Fas ligand interaction in colon cancer HT29 cells. *Carcinogenesis.* **23**: 2087–2093.
- Hedlund, M., M. Svensson, Å. Nilsson, R-D. Duan, and C. Svanborg. 1996. Role of the ceramide-signaling pathway in cytokine responses to P-fimbriated *Escherichia coli*. *J. Exp. Med.* **183**: 1037–1044.
- Bligh, E. H., and W. J. Dyer. 1959. A rapid method for total lipid extraction and purification. *Can J. Biochem. Physiol.* **37**: 911–918.
- Duan, R-D., and Å. Nilsson. 1997. Purification of a newly identified alkaline sphingomyelinase in human bile and effects of bile salts and phosphatidylcholine on enzyme activity. *Hepatology.* **26**: 823–830.
- Duan, R-D., Y. Cheng, H-D. Tauschel, and Å. Nilsson. 1998. Effects of ursodeoxycholate and other bile salts on levels of rat intestinal alkaline sphingomyelinase. A potential implication in tumorigenesis. *Dig. Dis. Sci.* **43**: 26–32.
- Duan, R-D., L. Nyberg, and Å. Nilsson. 1995. Alkaline sphingomy-

elinase activity in rat gastrointestinal tract: distribution and characterization. *Biochim. Biophys. Acta.* **1259**: 49–55.

37. Chatterjee, S. 1993. Neutral sphingomyelinase. *Adv. Lipid Res.* **26**: 25–48.
38. Hansen, G. H., E. M. Danielsen, H. Sjöström, and O. Norén. 1989. Organelles involved in the intracellular transport of newly synthesized aminopeptidase N and their acidity. *Eur. J. Cell Biol.* **49**: 154–161.
39. Hansen, G. H., L. L. Niel-Christiansen, M. D. Poulsen, O. Norén, and H. Sjöström. 1994. Distribution of three microvillar enzymes along the small intestinal crypt-villus axis. *J. Submicrosc. Cytol. Pathol.* **26**: 453–460.
40. Yang, L., M. Mutanen, Y. Cheng, and R-D. Duan. 2002. Effects of red meat and fiber in high fat diet on activities of sphingomyelinase, ceramidase and caspase-3 in rat colonic mucosa. *J. Nutr. Biochem.* **13**: 499–504.
41. Earnest, D. L., H. Holubec, R. K. Wali, C. S. Jolley, M. Bissonette, A. K. Bhattacharyya, H. Roy, S. Khare, and T. A. Brasitus. 1994. Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary ursodeoxycholic acid. *Cancer Res.* **54**: 5071–5074.
42. Colell, A., O. Coll, M. Mari, J. C. Fernandez-Checa, and J. Garcia-Valero. 2002. Divergent role of ceramide generated by exogenous sphingomyelinase on NF-kappa B activation and apoptosis in human colon HT29 cells. *FEBS Lett.* **526**: 15–20.