Sphingomyelin and phosphatidylcholine contrarily affect the induction of apoptosis in intestinal epithelial cells

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Scope: The major alimentary sources for the plasma membrane lipid sphingomyelin (SM) are dairy products, eggs, and meat. We recently reported that the SM metabolite ceramide induces cathepsin D mediated apoptosis in murine intestinal epithelial cells (IECs) and increases inflammation in acute colitis. We investigated the impact of SM and phosphatidylcholine on apoptosis in human IECs and point out BH3-interacting death agonist (BID) as link between cathepsin D and apoptosis.

Methods and results: HT–29 and isolated human IECs were stimulated with SM or phosphatidylcholine. SM treatment resulted in increased apoptosis. Phosphatidylcholine showed contrary effects. Western revealed higher amounts of cathepsin D and BID activation upon lipid stimulation. Western blotting revealed BID activation through SM in both an induced and a spontaneous mouse model of colitis.

Conclusion: Dietary phospholipids may induce or abolish apoptosis in IECs and seem to play a role in the pathogenesis of inflammatory bowel diseases. This nutritional factor might be considered when evaluating the pathogenesis of inflammatory bowel diseases. Effects of SMase- and SM treatment on inflammation in dextran sulfate sodium induced animal models of colitis and in vitro experiments are discussed as controversial. Variable sources of SM, feeding techniques, and mouse strains might play a role.

Keywords: Apoptosis / BID / Cathepsin D / Phosphatidylcholine / Sphingomyelin

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1 Introduction

The mechanisms of programmed cell death, or apoptosis, become imbalanced under pathologic conditions like inflammation [1]. In patients with inflammatory bowel disease (IBD), intestinal epithelial cells (IECs) die more frequently from apoptosis than in healthy individuals [2]. This is associated with an impaired integrity of the epithelial barrier and thought to be an important factor in the pathogenesis of ulcerative colitis (UC) [3] and Crohn’s disease (CD) [4].

Members of the B-cell lymphoma/leukemia-2 (BCL-2) protein family are well known to be involved in apoptotic events. They comprise prosurvival proteins such as BCL-2, BCL-xL, and myeloid cell leukemia sequence 1 (BCL2-related), as well as proapoptotic molecules, which are further
divided into two subfamilies. The first group includes Bax and Bak (where Bax is BCL2-associated X protein and Bak is BCL–2-antagonist/killer 1), expressing multiple regions of BCL–2 homology. The second group features only one domain of BCL–2, termed BH3, which gave rise to the subfamily’s name of BH3–only proteins [5]. BH3–only proteins are further classified into direct activators and sensitizers of apoptosis with BH3-interacting death agonist (BID) belonging to the first group [6, 7]. BID is cleaved, and thereby activated, to its truncated form truncated BID (tBID) by caspase-8 in response to Fas– [8, 9] or tumor necrosis factor (TNF) mediated death receptor-dependent apoptosis. tBID translocates from the cytosol to mitochondria where it activates BAX and BAK, both essential for mitochondrial apoptosis by forming pores in the mitochondrial outer membrane [10]. Cytochrome c is released from the mitochondrial intermembrane space into the cytosol where it contributes to the formation of the apoptosome [9, 11]. Subsequently, caspase–9 and –3 are activated and apoptosis is executed [12].

Recent data suggest cathepsins from lysosomes to act as novel mediators of cell death. Heinrich et al. described a central role for cathepsin D in an apoptotic pathway initiated by acid sphingomyelinase (aSMase) derived ceramide [13–15]. Ceramide was shown to activate cathepsin D and to mediate its translocation from lysosomes to the cytosol. Cathepsin D proteolytically generated active apoptogenic tBID. This resulted in cytochrome c release from mitochondria, activation of the caspase cascade, and apoptosis.

Ceramide is considered as the central hub of sphingolipid metabolism and as an important mediator in the signaling cascades involved in apoptosis [16]. One source of ceramide is dietary-derived sphingomyelin (SM), a lipid component of the plasma membrane being metabolized to ceramide by sphingomyelinases (SMases). In the Western diet, the main alimentary sources for SM are dairy products, eggs, and meat. In several cell types, ceramide has been implicated in differentiation [17], senescence [18], and apoptosis [19], whereof its function as proapoptotic molecule is probably most studied. Ceramide has been reported to release cytochrome c out of the mitochondrial intermembrane space [20] and to inactivate antiapoptotic BCL–2 [21].

Dietary SM is digested in the small intestine or colonic lumen [22], or reaches the colon via lymph [23] and serum. Sphingolipid consumption is estimated as 0.3–0.4 g per day per capita [24]. Dietary SM is poorly cleaved by pancreatic enzymes, but is hydrolyzed in all subsequent regions of the small intestine and colon by SMases with different pH optima depending on their location in the gastrointestinal tract [24]. Alkaline SMase plays a major role in breakdown of dietary SM. In addition, smaller amounts are contributed by the activity of aSMase [25]. Twenty-five percent of an administered dose (1–25 mg) of sphingomyelin is excreted in the feces, of which 10% is the intact molecule and 80–90% is the sphingolipid metabolite ceramide.

Also the phospholipid phosphatidylcholine (lecithin), being accessible via daily diet as well, was shown to play a role in apoptosis of different cell lines. Some evidence supports the idea that application of phosphatidylcholine may be a treatment option for UC. UC, but not CD, patients were shown to exhibit significantly lower concentrations of phosphatidylcholine in their ileal and colonic mucus [26]. Phosphatidylcholine protected the intestinal mucosa from acetic acid-induced colitis in rats [27], and the hypothesis of phosphatidylcholine having antiinflammatory effects was proven in patients with UC [28]. Stremmel et al. reported that UC patients benefit from orally applied retarded release of phosphatidylcholine that was shown to restore their mucosal barrier function [28].

We recently described that dietary SM, being metabolized to ceramide by SMases, triggers apoptosis in murine IECs and aggravates inflammation in acute dextran sulfate sodium (DSS) colitis [29]. Confirmative to those findings, colonic inflammation was increased in interleukin-10 knock-out (IL-10−/−) mice upon dietary SM [29]. In the present study, we demonstrate that SM initiates cathepsin D- and BID-mediated apoptosis in the human colonic cell line HT–29 and in primary IECs, whereas phosphatidylcholine protects the cells from death. Additionally, we point out BID as a potential link between cathepsin D and apoptosis during DSS colitis and SM treatment.

2 Materials and methods

2.1 Patients

Primary human IECs were obtained from surgical specimens from intestinal mucosa of patients undergoing surgery in the bowel (Supporting Information Table 1). This study was approved by the Cantonal Ethics Committee of Zurich (EK837) and performed according to the declaration of Helsinki.

2.2 Isolation of human colonic crypts and induction of apoptosis in IECs

IECs were isolated as described [30]. Human colonic mucosa from surgical specimens was cut into small strips (5 mm). Mucus was removed by incubating the strips for 30 min at room temperature in Hanks’ balanced salt solution (HBSS; Sigma-Aldrich, Switzerland) supplemented with 1 mM 1,4-dithiothreitol (DTT; Sigma-Aldrich). The strips were subsequently agitated in HBSS containing 1 mM EDTA (Sigma-Aldrich) for 10 min at 37°C, vigorously shaken for ten times and passed over a coarse mesh (400 µm, Carl Roth, Germany). Sieve throughput was discarded. Shaking and sieving was repeated three times and IECs in the thorough fractions were collected. The crypt- and single cell-containing suspension was filtered through a 70 µm cell strainer (BD Biosciences). Intact colonic crypts were retained in the cell strainer and eluted by inverting the cell strainer in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen, Switzerland) containing...
10% fetal bovine serum (PAA Laboratories, Germany). IEC suspensions were supplemented with 100 μM SM, 100 μM phosphatidylcholine (#85615 and #P3556, Sigma-Aldrich) or an adequate volume of water. Crypts were pelleted by centrifugation (600 g, 6 min, 4°C) and incubated for 2 h at 37°C or kept for 2 h at 37°C on an orbital shaker.

2.3 Induction of colitis and lipid treatment

Acute colitis was induced in female C57-BL/6J-Fue mice with 2% DSS over 7 days (the animal study was approved by the Cantonal Ethics Committee of Zurich, 146/2009). Four milligrams of SM, isolated from egg yolk as described previously [29], were resuspended in drinking water and daily applied by oral gavage. Animals were sacrificed on day eight. IL−10−/− mice daily received 4 mg SM or phosphatidylcholine (#85615 and #P3556, Sigma-Aldrich) in drinking water by oral gavage from postnatal week 11 to 14. The animals were sacrificed on day 22 of lipid treatment.

2.4 Isolation of murine IECs

Murine colon was sliced lengthwise and one half was used for isolation of IECs. After being freed from residual feces, the colon was cut into small pieces. IECs were loosened by shaking the mucosa in Hank’s balanced salt solution (HBSS; Sigma-Aldrich), supplemented with 2 mM EDTA (Sigma-Aldrich), for 30 min at 37°C. Mucosal pieces were separated from isolated crypts by passing the slurry over a coarse mesh (400 μm, Carl Roth). The sieve residue was transferred back to fresh HBSS, vigorously shaken for 20 times, vortexed briefly, and flushed through the mesh. Void-fraction was collected and shaking, vortexing and sieving steps were repeated twice. The collected cell suspension was passed over a 70 μm cell strainer (BD Bioscience, Belgium) to separate crypts from single IECs.

2.5 Preparation of liposomes and Nile red staining

Spray-dried SM or phosphatidylcholine (#85615 and #P3556; Sigma-Aldrich) were dissolved in MeOH:CHCl3 (1:1; v/v). The solvents were evaporated and the resulting lipid film was dried under vacuum. Lipids were resuspended in water, sonicated, and liposome formation was confirmed microscopically. Liposomes were either directly applied for stimulation or previously underwent Nile red staining and subsequent dialysis (using Slide–A–Lyzer MINI dialysis units (Thermo Fisher Scientific, Perbio Science, Switzerland; molecular weight cut off: 10 000)) to remove excess Nile red. For fluorescence microscopy, cells were mounted with medium containing 4′,6-diamidino-2-phenylindole’s emission was assigned to blue, Nile red emission was assigned to red.

2.6 Cell culture, immunofluorescence, Western blot, flow cytometry, TdT-mediated dUTP nick end labeling (TUNEL)

The methods were performed as described in the Supporting Information.

2.7 Lipid determination by ESI-MS/MS

Lipid extracts from cell homogenates, suspended in M-PER protein extraction buffer (Thermo Fisher Scientific) were prepared according to the method of Bligh and Dyer [31] in the presence of nonnaturally occurring internal standards. Quantification was performed by ESI-MS/MS in positive ion mode, using the analytical setup as previously described [32]. A precursor ion scan of m/z 184 specific for phosphocholine-containing lipids was used for SM [32]. Ceramide was analyzed similar to a previously described method [33] using N-heptadecanoyl-sphingosine as internal standard. Correction of isotopic overlap of lipid species as well as data analysis by self-programmed Excel Macros was performed for all lipid classes according to the principles described previously [32].

2.8 Sphingomyelinase activity assay in mouse tissue and HT-29

In mouse tissue, alkaline sphingomyelinase (alkSMase) activity was determined in the distal third of the jejunum. Samples were extracted with 150 mM NaCl (containing 6 mM sodium taurocholate) on a laboratory shaker (100 rpm, 1 h). The taurocholate provokes secretion of the enzyme from the tissue [34]. The tissue was removed, centrifuged (17 000 × g, 5 min, 4°C), and the supernatant was diluted tenfold with 100 mM Tris-HCl (pH 8). Activity was measured in duplicates using the Amplex® Red Sphingomyelinase Assay Kit (Molecular Probes, Invitrogen, Germany) according to the manufacturer’s instructions. Calculation of alkSMase activity needs to be adjusted: neutral sphingomyelinase (nSMase) activity is determined with the mentioned method for the same time. Thus, nSMase activity was examined in the samples extracted for determination of alkSMase and aSMase activities. The extracts were diluted tenfold with Tris-HCl (pH 7.4). Measurement was performed in duplicates using the Amplex Red Sphingomyelinase Assay Kit according to the manufacturer’s instructions. nSMase activity was subtracted from the previously obtained results for alkSMase activity to obtain the adjusted alkSMase activity.

aSMase activity was determined in colonic tissue. Samples were suspended in 100 mM Tris-HCl, homogenized with an ultra turrax and undergone three freeze-thaw cycles. After removal of the tissue and centrifugation (17 000 × g, 5 min,
4°C), the supernatant was diluted tenfold with 50 mM sodium acetate (pH 5). Activity was measured in duplicates using the Amplex® Red Sphingomyelinase Assay Kit (Molecular Probes) according to the manufacturer’s instructions. Calculation of aSMase activity was also adjusted for nSMase activity as described above.

In HT–29, a similar procedure was applied. Cells were suspended in 150 mM NaCl containing 6 mM sodium taurocholate and underwent three freeze-thaw cycles. After centrifugation (17 000 × g, 5 min, 4°C), the supernatant was diluted tenfold with the respective buffers described for mouse tissue. Measurement was performed in duplicates using the Amplex Red Sphingomyelinase Assay Kit according to the manufacturer’s instructions.

2.9 siRNA transfections

Transient downregulation of cathepsin D expression in HT–29 was achieved using Nucleofector® kit V (Amaza, USA). Specifically, 10⁶ cells per condition were suspended in 100 μL of Nucleofector® solution and 6 μL of 50 μM siRNA mix containing three different siRNA sequences: s135, s136, s137 (Life technologies/Ambion, USA) at RT. Next, the samples were subjected to nuclease using high-efficiency protocol (V001) and grown in media for 24 h. Next, cells were transferred to starvation medium (medium without FCS) for 48 h.

2.10 Statistical analyses

Statistical analyses were performed using PASW statistics 18.0 (SPSS, USA). Kruskal–Wallis nonparametric analysis of variance and Bonferroni-corrected Mann–Whitney rank sum test were applied for animal experiments and analysis of human samples. Box plots express median, 25% quartiles around median, minimum, and maximum. One-Way ANOVA and Tukey posthoc tests were used for cell culture experiments. Bars represent mean values with Whiskers displaying SD. Differences were considered significant at

\[ p < 0.05 (\*) \]

highly significant at \[ p < 0.01 (^{*}{*}) \] and very highly significant at \[ p < 0.001 (^{*}{*}{*}{*}) \]. Luminescence of Western blots was quantified densitometrically with OptiQuant (Packard Instrument, USA).

3 Results

3.1 SM is metabolized to ceramide in HT–29 after liposomal administration

As experimental evidence suggests an important role for ceramide in inflammation and cell death, we focused on the involvement of SM-derived ceramide in the inflammatory process and on its property to induce apoptosis in IECs. We analyzed, whether freshly generated SM and phosphatidylcholine liposomes were taken up by HT–29. Successful formation of liposomes was confirmed microscopically (Supporting Information Fig. 1A and B). To test whether the lipids were taken up into HT–29, we stained the liposomes with Nile red, removed excess dye by 2 h of dialysis, and applied the lipids to the cells. Nile red staining persisted during dialysis (Supporting Information Fig. 1C–F). As Nile red is an unspecific lipophilic dye, it can also bind endogenous ceramide and other lipids (Supporting Information Fig. 1G and H). To exclude unspecific staining, we incubated HT–29 with supernatant of dialysis containing excess dye. No fluorescence was detected (Supporting Information Fig. 1k and L). Increased uptake of stained and dialyzed SM liposomes by HT–29 was shown after 2, 18, and 24 h compared to 1 h and the negative control (Supporting Information Fig. 2A–E). After 24 h, both SM and phosphatidylcholine liposomes were localized inside the cells (Fig. 1A, a–c). As proven by ESI-MS/MS quantification, lipid uptake reached a higher level for both SM and phosphatidylcholine when HT–29 underwent prestimulation with interferon gamma (IFN–γ) (50 ng/mL) for 12 h and, subsequently, TNF (2.5 ng/mL) for 24 h (Fig. 1B and C). SM was metabolized to ceramide, as clearly more ceramide could be detected by immunofluorescence after 48 h of 100 μM liposomal SM (Fig. 2A, a and b) compared to unstimulated cells (Fig. 2A, c or application of 100 μM phosphatidylcholine (Fig. 2A, d and e). Quantification of cellular ceramide by ESI–MS/MS revealed a highly significant increase in ceramide levels after SM stimulation (Fig. 2B).

3.2 Cathepsin D and truncated BID (tBID) protein levels are increased in HT–29 after administration of SM

Recent data suggest that elevated ceramide levels induce apoptosis via activation of cathepsin D and BID. We analyzed pro-, intermediate, and mature cathepsin D as well as cleavage of BID to tBID in HT–29 by Western blot. Total amounts of cathepsin D were increased upon 48 h of stimulation with 100, 10, or 1 μM SM (1.2-fold each, compared to untreated HT–29; \( n = 3 \) independent experiments, Fig. 3A). Also the level of pro- and intermediate cathepsin D was augmented (1.4-fold, 1.4-fold, and 1.5-fold for 100, 10, and 1 μM, respectively, compared to untreated HT–29; \( n = 3 \) independent experiments). As positive control for cathepsin D activation, HT–29 were incubated with 100 ng/mL TNF for 48 h. Total quantity and the amount of pro- and intermediate cathepsin D were increased (1.2-fold and 1.4-fold, respectively; \( n = 3 \) independent experiments). tBID was elevated upon 48 h of stimulation with 100 μM SM (2.8-fold, compared to untreated HT–29; \( n = 3 \) independent experiments, Fig. 3A). Compared to untreated HT–29, tBID levels were augmented 3.8-fold upon 100 ng/mL TNF for 48 h (\( n = 3 \) independent experiments).
BID activation was confirmed by immunofluorescent staining for tBID. Stimulation of HT–29 for 48 h with 100 μM of liposomal SM resulted in a clear increase of proapoptotic tBID levels (4.9-fold, respectively, as compared to control; Fig. 3B, a and c).

### 3.3 SM induces apoptosis in HT–29 whereas phosphatidylcholine protects from cell death

To determine whether SM-driven cathepsin D activation and cleavage of BID affects apoptosis, HT–29 were stimulated with liposomal SM (100 μM), phosphatidylcholine (100 μM), or water for 24 h. Cells either underwent prestimulation with IFN–γ (50 ng/mL) for 12 h and, subsequently, TNF (2.5 ng/mL) for 24 h to mimic the inflammatory conditions present during CD and UC, or remained without prestimulation. Numbers of living and apoptotic cells were determined by flow cytometry of annexin V- and PI-stained cells. Prestimulation with IFN–γ and TNF significantly decreased the number of living cells by 28 ± 20% compared to unstimulated HT–29 (n = 3 independent experiments, p < 0.05, not shown). The values for water-stimulated controls were set to 100%. Without prestimulation, SM, and phosphatidylcholine did not influence the fractions of living and apoptotic cells (n = 4 independent experiments; Fig. 4A and B). In contrast, following IFN–γ and TNF prestimulation, SM amplified the cytokine-induced decrease in living and increase in apoptotic cells (living: 92 ± 8%, n = 3; apoptotic: 177 ± 66%, n = 3; Fig. 4A and B and Supporting Information Fig. 3A). Unlike SM, phosphatidylcholine prevented apoptosis in IFN–γ and TNF pretreated HT–29 (living: 112 ± 10%, n = 3; apoptotic: 65 ± 15%, n = 3; Fig. 4A and B and Supporting Information Fig. 3A). When comparing SM to phosphatidylcholine-treated prestimulated HT–29, significantly fewer cells remained alive and significantly more cells were in the state of early apoptosis due to SM application (p < 0.05; Fig. 4A and B and Supporting Information Fig. 3A). Further, in HT-29 cultured in medium without FCS, SM significantly amplified the starvation-induced decrease in living and increase in apoptotic cells (living: 94 ± 3.6%, n = 6 independent experiments; apoptotic: 119 ± 21%, n = 6; p < 0.05, Fig. 4A and B). Phosphatidylcholine did not significantly shift the fractions of living and apoptotic cells (living: 98 ± 2.9%, n = 6; apoptotic: 108 ± 12.8%, n = 6; Fig. 4A and B).

The observed effects were found to be dependent on the applied amount of lipids. In IFN–γ and TNF prestimulated HT–29, SM, and phosphatidylcholine needed to be applied at a minimal concentration of 10 μM to induce or prevent apoptosis, respectively (Supporting Information Fig. 3B and C). Application of 100 μM SM further increased the number of apoptotic cells (Supporting Information Fig. 3C). Additionally, we found that prestimulation of HT–29 with IFN–γ and TNF significantly increased the activity of alkSMase and nSMase to 134 ± 16% and 133 ± 10% of unstimulated controls, respectively (n = 3 independent experiments, p < 0.05; Fig. 4C).
To confirm whether cathepsin D could modify SM-induced apoptosis in HT-29, we performed cathepsin D knock-down studies using siRNA. We transfected HT-29 with either nonspecific siRNA or cathepsin D-specific siRNA constructs. After 24h, we subsequently treated these cells with SM (100 μM) for 48h. Transfection with cathepsin D specific siRNA caused a maximal decrease in cathepsin D protein of 65.0 ± 7.0% compared to transfection with control siRNA (Supporting Information Fig. 3D, n = 6 independent experiments). Western blot of the loading control, β-actin, demonstrated that the transfection procedure itself did not affect the overall protein level in our cells (Supporting Information Fig. 3D). We then tested whether cathepsin D knock-down could affect SM-induced apoptosis. As cathepsin D links TNF-induced aSMase to apoptosis, absence of cathepsin D may also reduce TNF-mediated effects. Therefore, we used starvation to sensitize cells to undergo apoptosis. SM treatment decreased cell survival in control siRNA cells (94.1 ± 6.0%, n = 6 independent experiments), and loss of cathepsin D prevented this effect (99.1 ± 1.9%, n = 6 independent experiments, Fig. 4D). These findings demonstrate that cathepsin D regulates the initiation of apoptosis in response to SM in intestinal epithelial cell lines.

3.4 Liposomal SM and phosphatidylcholine contrarily affect the induction of apoptosis in human primary IECs

We investigated, whether induction of apoptosis upon SM and protection from cell death upon phosphatidylcholine is also detectable in ex vivo isolated human IECs from surgical specimen. One fraction of the cells was fixed directly after isolation. Other fractions were either stimulated with 100 μM liposomal SM, phosphatidylcholine, or water. As ex vivo isolated IECs are usually inert upon stimulation compared to cell lines and liposomes above 100 μM are not reliable to prepare, we used the highest maintainable dose for
SM stimulation identified in cell culture experiments (Fig. 3). The fractions were pelleted by centrifugation and incubated at 37°C for 2 h or remained in suspension by permanent agitation at 37°C for 2 h. Significantly increased levels of SM and phosphatidylcholine (Supporting Information Fig. 4A, a and b) as well as significant metabolism of SM to ceramide (Supporting Information Fig. 4A, c) were proven by ESI–MS/MS.

Freshly isolated colonic crypts were stained with TUNEL reagent to determine DNA strand breaks. 18.9 ± 10.4% of the IECs were TUNEL positive and therefore apoptotic (n = 6 independent experiments; Fig. 5A, a and d). The number of apoptotic cells significantly increased after 2 h in pelleted IECs (50.6 ± 17.1%, n = 6, p < 0.05). Apoptosis further increased significantly when the cells were kept in suspension for 2 h (81.6 ± 11.7%, n = 6, p < 0.05; Supporting Information Fig. 4B). Representative images of TUNEL stained control IECs, incubated for 2 h at 37°C, either as pellet or in suspension, are shown (Fig. 5A, b and c and e and f, n = 6 independent experiments). In suspended cells, fragmentation of nuclei was visible (Fig. 5A, c and f).

When setting the number of apoptotic cells of water-stimulated controls to 100%, SM (100 μM) significantly increased cell death in pelleted IECs (125.8 ± 28.2%, n = 6 independent experiments, p < 0.01; Supporting Information Fig. 4C). In contrast, phosphatidylcholine (100 μM) highly significantly reduced the number of apoptotic IECs in cells kept in suspension (91.9 ± 4.5%, n = 6, p < 0.01; Supporting Information Fig. 4C). Additionally, the cells were stained with PI and analyzed by flow cytometry. Confirmative to the TUNEL assay, 7.1 ± 3.6% of the freshly isolated IECs were found in the sub–G1 fraction and were therefore considered to be dead or dying (n = 10 independent experiments; Supporting Information Fig. 4D). This proportion significantly increased after 2 h in pelleted IECs (31.2 ± 12.6%, n = 10; p < 0.001) and was further elevated significantly when the IECs remained in suspension for 2 h (44.1 ± 12.4%, n = 10, p < 0.001; Supporting Information Fig. 4D).

When setting the sub–G1 fractions of water-stimulated controls to 100%, SM (100 μM) significantly increased cell death each in pelleted and suspended IECs of specimens.
obtained from control and IBD patients (pellet, non-IBD: 114.7 ± 17.5%, n = 5, p < 0.05; pellet, IBD: 111.9 ± 13.0%, n = 4, p < 0.05; suspension, non-IBD: 108.5 ± 4.4%, n = 5, p < 0.01; suspension, IBD: 103.5 ± 6.1, n = 4, p > 0.05; Fig. 5B). In contrast, phosphatidylcholine (100 μM) reduced the number of IECs in the sub–G1 fraction in cells kept both as pellet and in suspension. The decrease was highly significant for suspended IECs from control patients (97.1 ± 2.9%, n = 4, p < 0.01; Fig. 5B).

3.5 Dietary SM increases the level of intracellular ceramide in control mice and during acute DSS colitis

Recently, we reported the induction of apoptosis in murine IECs upon dietary SM. Here we examined whether apoptosis was a consequence of increased ceramide levels upon dietary SM. An immunofluorescent staining for ceramide in colonic tissue of a control mouse without DSS and without dietary SM is shown in Fig. 6A (representative image for n = 11). Dietary SM was applied to control mice without DSS treatment. The animals received 4 mg SM every day for 7 days. The lipid was resuspended in drinking water and applied by oral gavage. As seen by confocal microscopy, ceramide levels clearly increased when mice received SM in healthy controls (Fig. 6B, representative image for n = 6). An immunofluorescent staining for ceramide in colonic tissue of a mouse with DSS and without dietary SM is shown in Fig. 6C (representative image for n = 17). Dietary SM was applied to mice undergoing DSS treatment for induction of acute colitis. Ceramide levels clearly increased when mice received SM in DSS-treated animals (Fig. 6D, representative image for n = 12). This is likely due to metabolic hydrolysis of SM by SMases. nSMase was activated during DSS application in the colon but not in the jejunum, independent of mice receiving 4 mg SM or not (Supporting Information Fig. 5A and B). In contrast, colonic aSMase was activated by SM treatment, but not by DSS (Supporting Information Fig. 5C). Jejunal alkSMase was neither affected by SM nor by DSS application (Supporting Information Fig. 5D).

3.6 DSS and SM treatment activates BID

To confirm that ceramide-initiated apoptosis is mediated via BID activation, we determined tBID mRNA and protein expression upon dietary SM during acute DSS colitis. Relative Bid mRNA levels were neither affected by oral gavage of SM nor by DSS application (Supporting Information Fig. 6A...
SM-induced apoptosis in isolated human colonic crypts, whereas phosphatidylcholine prevented cell death. (A) TUNEL analysis in isolated human colonic crypts of six patients. Representative images of a) + d) freshly isolated crypts and b) + e) crypts kept as pellet or c) + f) in suspension for 2 h at 37°C. Cells were mounted with medium containing 4',6-diamidino-2-phenylindole. 4',6-diamidino-2-phenylindole's emission was assigned to blue, TUNEL+ cells are shown in green. (B) Flow cytometric cell cycle analysis of PI-stained isolated human colonic crypts of control (n = 5) and IBD (n = 4) patients, stimulated with water (c), liposomal SM (100 μM) or phosphatidylcholine (100 μM). Crypts were kept as pellet or in suspension for 2 h at 37°C. Apoptosis of control crypts was set to 100%. Representative histograms are shown; bars represent sub-G1 fraction used for quantification.

3.7 BCL–2 protein levels are decreased during DSS colitis and in response to dietary SM

As BID, a proapoptotic member of the BCL–2 protein family, was influenced by dietary SM and phosphatidylcholine, we wondered, whether also antiapoptotic BCL–2 itself was altered upon lipid treatment. When the animals received DSS for induction of an acute colitis, BCL–2 protein levels were significantly decreased (p < 0.05; Fig. 8A). The same is true for oral gavage of SM, as also mice receiving 4 mg of the lipid per day exhibited a significant drop in BCL–2 in their colonic tissue (p < 0.05; Fig. 8A). When the SM treatment was combined with the induction of an acute colitis, no additive effects concerning further decreased concentration of BCL–2 were detected. Also in IL–10−/− mice, dietary SM, but not phosphatidylcholine, resulted in significantly reduced levels of BCL–2 protein in colonic tissue (p < 0.05 for SM; Fig. 8B).
3.8 Tight and adherens junctions are weakened upon dietary SM and strengthened upon phosphatidylcholine

For evaluation of the epithelial integrity, tight and adherens junction protein levels were determined by Western blot and immunofluorescence. In IL–10−/− mice, dietary SM led to increased apoptosis in colonic tissue, as caspase–3 was significantly activated compared to IL–10+/− mice receiving water by oral gavage (Fig. 9A). Phosphatidylcholine did not influence the activation of caspase–3. The content of the tight junction protein occludin remained unchanged in colonic tissue when the animals received SM, but was significantly augmented upon phosphatidylcholine (p < 0.05 for phosphatidylcholine; Fig. 9B). Also adherens junctions were altered, as, in response to dietary phosphatidylcholine, β-catenin and E-cadherin protein concentrations were significantly elevated in colonic tissue, respectively (p < 0.05 for phosphatidylcholine compared to control or dietary SM, for β-catenin and E-cadherin; Fig. 9C and D). Immunofluorescence revealed that, upon dietary SM, E-cadherin was only found at the bottom of the colonic crypts (arrows, Fig. 9E, b, enlargement, lower lane), but mainly lacked in the extrusion zone where cells are expected to be shed (Fig. 9E, b, enlargement, upper lane). In contrast, in mice receiving water or phosphatidylcholine by oral gavage, fluorescence for E-cadherin was detected all along the crypt-villus axis (arrows, Fig. 9E, a and c, enlargements lower and upper lane).

Western blot proved that the relative amount of tight junctional pore-forming protein claudin–2 was significantly increased in mice upon SM as compared to water control (related to β-actin, Fig. 9F). Immunofluorescence confirmed that even in mice without DSS, dietary SM caused an increase in claudin–2 levels in comparison to mice receiving water by oral gavage (Fig. 9G).

4 Discussion

Apoptosis of IECs more frequently occurs in patients with IBD than in healthy individuals [2–4]. Cell death leads to an impaired integrity of the epithelial barrier and is thought to be a major factor in the pathogenesis of IBD [4, 35–38]. It has been suggested that the protease cathepsin D acts as a novel mediator of programmed cell death [13] and that activation of this enzyme can be induced by ceramide [14, 15]. Recently, we reported that dietary SM and its main metabolite ceramide induce cathepsin D mediated apoptosis in murine IECs and increase inflammation in acute DSS colitis [29]. In contrast, the structurally related phospholipid phosphatidylcholine was shown to restore the mucosal barrier function in UC [39]. We, therefore, studied the impact of SM and phosphatidylcholine on apoptosis in HT–29 and in isolated human IECs. We present activation of BID as a link between cathepsin D and cell death during dietary SM in DSS colitis.
Bid is activated to tBid in response to applied sphingomyelin (SM) and/or DSS treatment in murine IEC. (A) Immunofluorescent staining for tBid in murine colonic tissue. Tissue was mounted with medium containing 4',6-diamidino-2-phenylindole. 4',6-diamidino-2-phenylindole’s emission was assigned to blue, immunofluorescent staining for tBID was assigned to purple. Mice without DSS and either without dietary SM (representative image for n = 11) or with 4 mg SM a day (representative image for n = 6). d Negative control, without primary antibody. (B–E) Western blot analysis showing activation of BID to tBID. (B) IEC of C57BL/6J-Fue mice treated without or with DSS and without or with 4 mg SM. Homogenized colonic tissue of C57BL/6J-Fue mice treated (C) without DSS and without or with 4 mg dietary SM or (D) with DSS and without or with 4 mg SM. (E) Homogenized colonic tissue of IL–10–/– mice receiving water (c), 4 mg SM or 4 mg phosphatidylcholine. *p < 0.05, **p < 0.01.

Within the present study, we demonstrate an SM-mediated increase of apoptosis in HT–29. Stimulation with liposomes augmented the intracellular amount of cathepsin D and activated BID to tBID, but did not affect cell death of HT–29. HT–29 are derived from a human colon adenocarcinoma and express a number of oncogenes including K-Ras, H-Ras, and c-myc. p53 is mutated. This explains the poor responsiveness of HT–29 to weak apoptotic stimuli such as SM-derived ceramide. Since TNF expression is increased during IBD [40], a prestimulation with the cytokine might mimic the inflammatory conditions in patients. It furthermore induces both apoptosis and cell shedding in mice [41]. Additionally, HT–29 are more susceptible to TNF and apoptosis after IFN–γ prestimulation [42]. When IFN–γ and TNF preincubation preceded liposome stimulation, SM increased cell death in HT–29 in a dose-dependent manner, whereas phosphatidylcholine was found to exhibit antiapoptotic features. Most likely, this prestimulation renders the cells accessible to the lipid stimuli caused by activation of SMases that was described earlier [43] and within the present study. SMases metabolize SM to bioactive ceramide that triggers apoptosis. Correspondingly, in mice, dietary SM increased apoptosis of IECs when the animals received DSS, but did not alter the amount of apoptotic cells in control mice [29]. In this work, we show the activation of colonic nSMase upon DSS treatment. nSMase catalyzes the cleavage of the phosphodiester bond in SM, yielding ceramide and phosphorylcholine [44]. An increased nSMase activity therefore leads to a higher metabolization rate of SM, resulting in more proapoptotic ceramide that induces cathepsin D- and BID-mediated apoptosis.

Effects of SMase- and SM treatment on inflammation in DSS-induced animal models of colitis are discussed controversial. Andersson et al. described effects of intrarectal instillation of alkSMase produced from yeast cells on DSS-induced colitis in rats [45]. Intrarectal instillation of alkSMase significantly reduced the inflammation score and protected the...
colonic epithelium from inflammatory destruction. In contrast, increased expression for inflammatory marker IL-6 was determined despite the lower grade of histologic inflammation. Furuya et al. showed that dietary SM alleviates DSS-induced colitis [46]. Commercially available SM was supplemented with 0.1% w/w to the feeding of mice for 10 days and acute colitis was induced from day four. The authors demonstrated an increase of body weight and an improved histological score after dietary SM in Jcl:ICR mice with DSS colitis. In contrast, Sakata et al. described blocking of aSMase-catalyzed ceramide production with the inhibitor SMA-7 [47]. Orally administered SMA-7 reduced DSS-induced intestinal inflammation in mice. Interestingly, epithelial preservation was significantly improved [47]. Reduced DSS-induced intestinal inflammation in mice upon SMA-7 was confirmed in an additional study [48]. Interestingly, administration of SMA-7 significantly reduced the crypt damage and improved the regeneration of the epithelial layer. It is extremely difficult to relate experiments, but variable sources of SM as well as feeding techniques and mouse strains might play a role.

When isolating colonic crypts from human bowel resections, the obtained IECs rapidly died. Ex vivo isolation of colonic crypts might also increase the total number of apoptotic cells due to the partial loss of cell–cell contacts, cell–matrix contacts and inevitable physical stress or it also might result in prestimulation of cells. Pelleting of crypts maintains cell–cell contacts in IECs and prevents apoptosis [49]. In pelleted IECs, SM led to a stronger relative increase in apoptosis when compared to suspended IECs, whereas the contrary situation is true for phosphatidylcholine: it more potently inhibits cell death when the apoptotic stimulus is most pronounced. It has been shown that UC patients benefit from high doses of retarded release phosphatidylcholine, since it restores the phospholipid being reduced in their gut mucus [26, 28].

Heinrich et al. suggested SMase-derived ceramide to activate the protease cathepsin D [14]. The enzyme gets released from lysosomes, cleaves BID to active tBID, and initiates cytochrome c-mediated apoptosis in HeLa cells and fibroblasts [13]. In our recent work, SM aggravated an acute experimental colitis and increased apoptosis in DSS-receiving mice via activation of cathepsin D [29]. SM gavage activated cathepsin D in both mice receiving DSS and water control animals. In contrast, dietary SM led to increased apoptosis of IECs in animals with acute colitis only [29]. We investigated, whether BID was also involved. In accordance to the pathway suggested by Heinrich et al., SM also augmented the tBID/BID ratio in healthy control animals. In contrast, BCL-2 protein levels remained unchanged in IECs upon dietary SM compared to water control mice emphasizing the role of BID. Application of DSS did not alter the amounts of active cathepsin D during the induction of an acute colitis [29], but we determined significantly increased levels of tBID. Costimulation with SM during an acute DSS colitis did not further affect the tBID/BID ratio. The significant increase of tBID caused by DSS treatment might thereby mask the SM stimulus. In accordance to the increased apoptosis upon SM, BCL-2 protein levels were reduced upon dietary SM and upon DSS treatment. An additive effect was not evident. Interestingly, SM was highlighted to suppress inflammation-driven colon cancer in mice [50] which might be consistent with our results on SM increasing apoptosis. In the study presented here, we could show that colonic aSMase was activated by SM treatment. This is in agreement with in vitro observations demonstrating that sphingolipid metabolites lower Bcl-2 and thereby facilitate apoptosis [51]. In contrast, Simon et al. showed a significant increase in Bcl-2 protein expression in colonic mucosa upon SM (from a lipid whey extract, 0.05% supplement by weight, [52]).

However it is still controversial whether intestinal inflammation is increased upon SM. Mazzei et al. demonstrated upregulated gene expression levels of IFN-γ, IL-17, and IL-23 in acute DSS induced colitis upon SM treatment [50]. Differentiation of CD4+ T cells toward Th1
Figure 9. Adherens and tight junctions are weakened upon dietary SM and strengthened upon phosphatidylcholine. (A–D) Western blot analysis of homogenized tissue of IL–10−/− mice receiving water (c), 4 mg SM or 4 mg phosphatidylcholine. (A) Pro- and active caspase–3. (B) Occludin. (C) β-Catenin. (D) E-cadherin. (E) Immunofluorescence for E-cadherin in colon of IL–10−/− mice receiving a’ water (c), b’ 4 mg SM or c’ 4 mg phosphatidylcholine. Tissue was mounted with medium containing 4,6-diamidino-2-phenylindole. 4,6-diamidino-2-phenylindole’s emission was assigned to blue, immunofluorescent staining for E-cadherin was assigned to red (representative image for n = 5 each). (F) Western blot analysis for claudin–2 in homogenized colonic tissue of C57BL/6J-Fue mice received drinking water without DSS. Mice were treated without and with 4 mg dietary SM. (G) Immunofluorescence for claudin-2 of C57BL/6J-Fue mice received drinking water without DSS. Mice were treated without and with 4 mg dietary SM. Immunofluorescent staining for claudin-2 was assigned to red (representative image for n = 5 each).

(by IFN-γ) and Th17 (by IL-17 and IL-23) effector phenotypes is involved in cellular responses against pathogens and autoimmunity such as IBD. Further, a number of gut homing chemokines and chemokine receptors were upregulated in acute DSS-induced colitis upon SM treatment. The majority of genes upregulated upon SM are immunostimulatory and proinflammatory, which is in agreement with results from previous studies demonstrating the proinflammatory effect of sphingolipid metabolites [53–57]. On the other hand, upregulation of genes in line with an anti-inflammatory effect like the Treg-related gene IL-10R was also found upon this treatment. In the mentioned study, the sphingolipid group received 1 g/kg SM powder from bovine milk in the diet compared to control mice fed with semipurified sphingolipid-free diet alone (n = 3 each). The authors conclude that SM induced a proinflammatory response that was paralleled by an anti-inflammatory immune response. This gene expression pattern was associated with decreased colonic inflammatory lesions. The authors suggested a regulatory response may have paralleled the proinflammatory gene expression pattern.

This is not in accordance with our former results [29] and with the data presented here. The genetically driven absence of Tregs in the IL–10−/− mice used in our own experiments could explain the induction of a proinflammatory response upon SM. However in our own experiments, dietary SM also aggravated mucosal damage upon DSS-induced colitis in fully immune competent wild-type mice [29]. Variable sources of SM, different feeding techniques, and mouse strains might also serve for explanation. In the study presented here SM,
isolated from egg yolk, was used as described previously [29]. SM, isolated from egg yolk has a different fatty acid (FA) profile (86.6% C16, 3.0% C22, not detectable C23 and 3.0% C24 FA) compared to SM isolated from milk (23.4% C16, 18.2% C22, 30.0% C23, and 17.7% C24 FA) [58]. This affects lipid absorption [58] and amid-bound fatty acid is thought to have a crucial impact on cellular responses.

The different fate of diet-derived ceramide with varying FA compositions in the intestine may be a crucial factor. SM incubated with colonic segments from mice disappeared during incubation and increases in ceramide were apparent [59]. It has been shown that after the digestion of milk sphingolipids, the major ceramide species accumulating in the intestine is the C16-ceramide although not a dominant species
in milk sphingolipids. It is feasible that the administration of C16-SM isolated from egg yolk per oral gavage leads to high levels of C16-ceramide that induce apoptosis while these levels may be lower after administration of SM isolated from milk.

Decrease in adherens junctions [60, 61] and loss of tight junctions [62] are connected to increased apoptosis in intestinal epithelial cells. Further, decrease in adherens [63] and tight junctions [64] are connected to loss of barrier function due to increased apoptosis during chronic inflammation. An impaired intestinal barrier function in patients with active CD [65] and in UC [66] is connected with a strong upregulation of the pore-forming tight junctions protein claudin-2. Upregulation of claudin-2 leads to an increased paracellular permeability [67]. Furthermore, upregulation of claudin-2 could be associated with increased apoptosis of IECs as the proinflammatory cytokines TNF [68, 69], IFN-γ [68], and IL-13 [69] initiate apoptosis and increase claudin-2 protein expression in HT-29 [69] and native rat colon [68]. In our study, adherens and tight junctions also seem to play a role. Alterations in the junctional complexes may be involved in the effects caused by phosphatidylcholine, as they were found to be strengthened due to increased levels of occludin, β-catenin, and E-cadherin. On the other hand, claudin–2 was increased during dietary SM.

Taken together, our findings provide evidence that SM increases apoptosis in human IECs, whereas phosphatidylcholine protects from cell death. The SM-related effects are mediated by ceramide-induced activation of cathepsin D and subsequent cleavage of BID to tBID, and further involve BCL–2. Thus, the sphingolipid amplifies apoptosis of IECs linking ceramide activated cathepsin D to the mitochondrial apoptotic TNF pathway as suggested by Heinrich et al. [14].

As apoptosis is increased during IBD, cell death influencing dietary phospholipids seem to play a role in the pathogenesis of intestinal inflammation. They involve diverse signaling mechanisms and influence the course of the diseases positively or negatively. The daily consumption of SM and phosphatidylcholine strongly depends on personal nutritional habits and on the ingested amount of animal-derived food. This nutritional factor should be considered when evaluating the pathogenesis and perpetuation of IBD.

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5 References


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