Metabolism and apoptotic properties of elevated ceramide in HT29^{rev} cells

Robert J. VELDMAN, Karin KLAPPE, Dick HOEKSTRA and Jan W. KOK¹

Department of Physiological Chemistry, University of Groningen, Antonius Deusinglaan 1, 9713 AV, Groningen, The Netherlands

Ceramide (Cer) has been implicated in the regulation of apoptosis. In this study, we elevated cellular Cer levels in human colon-carcinoma (HT29^{rev}) cells by incubating the cells in the presence of bacterial sphingomyelinase (bSMase) or, alternatively, in the presence of C₂-Cer, a short-chain analogue of the sphingolipid. bSMase treatment did not induce apoptosis in these cells, as revealed by a lack of both DNA fragmentation and cleavage of poly(ADP-ribose)polymerase. In contrast, apoptosis did occur upon addition of C₂-Cer. These findings led us to study whether differences in the metabolic fate of the excess of Cer, as generated by both treatments, contributed to the observed difference in apoptosis-inducing capacity. C₂-Cer was rapidly taken up by HT29^{rev} cells and accumulated due to the absence of substantial metabolic conversion. Upon addition of bSMase, hydrolysis of sphingomyelin resulted in a reduction of that pool to 20 % compared with control values, accompanied by a multi-

fold increase in Cer level. In spite of the continuous presence of active bSMase, the Cer increase turned out to be transient. Cer levels reached their maximum 1–2 h after addition of bSMase, followed by a significant decrease. Excessive Cer was mainly turned over via cerebrosides into complex glycolipids, including gangliosides. In the presence of glucosylceramide synthase- and/or ceramidase inhibitors, this conversion was significantly blocked and bSMase-generated Cer accumulated in the cells. However, even under these conditions apoptosis did not occur. In conclusion, the inability of bSMase to induce apoptosis of HT29^{rev} cells does not appear to be due to rapid metabolic conversion of excessive Cer. Since apoptosis is induced upon addition of C₂-Cer, we therefore propose that the intracellular target involved in the propagation of the apoptotic signal is reached by C₂-Cer, but not by bSMase-generated Cer.

INTRODUCTION

The generation of ceramide (Cer) has been implicated in signal transduction pathways leading to various cellular responses, including programmed cell death [1–3]. In the apoptotic response, Cer mediates the cytotoxic actions of extracellular ligands such as Fas/Apo-1 [4,5] and tumour necrosis factor α [6], or stress stimuli such as ionizing radiation [7]. In addition, cytotoxic drugs, such as daunorubicin, are reported to exert their action via this Cer-mediated mechanism [8]. Sphingomyelin (SM), a sphingolipid abundantly present in the plasma membrane, is generally the source of signalling-involved Cer. After hydrolysis of SM by either a neutral [9] or an acidic sphingomyelinase (SMase) [10], Cer levels increase, which in turn triggers signalling cascades, eventually leading to apoptosis. The direct cellular targets of Cer are presumably a proline-directed kinase [11,12], a serine/threonine protein phosphatase [13] or protein kinase C ζ [14].

We have previously reported that compared with other cells, undifferentiated human colon HT29 G⁺ cells exhibit a high level of neutral SMase activity in the exoplasmic leaflet of the plasma membrane, which is responsible for rapid turnover of membrane-inserted C₆-7-nitro-2,1,3-benzoxadiazol-4-yl-SM [15]. In spite of this Cer-generating capacity, HT29 cells are known to be generally resistant to drug-induced cell death [16]. Differentiated human colon-carcinoma (HT29^{rev}) cells express a much lower activity of neutral SMase, which may be related to a higher susceptibility to Cer-induced cell death.

In this study we used bacterial SMase (bSMase) to rapidly hydrolyse all accessible SM and thereby cause a multi-fold increase in the cellular Cer level. In several cell types this

procedure results in the onset of apoptosis [2,5]. However, HT29^{rev} cells were resistant to bSMase-induced cell death. Culturing of HT29^{rev} cells in the presence of a short-chain analogue of Cer, on the other hand, rapidly induced apoptosis, in a specific manner, since C₂-dihydroceramide (C₂-DH-Cer) was ineffective. The reason for the biological inactivity of Cer generated in the exoplasmic leaflet of the plasma membrane is unknown, but may be related to fast metabolic turnover of Cer.

In the present study therefore, we investigated the cellular response to elevated Cer in terms of its metabolic processing. Our data indicate that remarkable differences exist in the way that bSMase-generated Cer is metabolized, when compared with C₂-Cer, and new insight is gained with respect to the metabolic fate of excessive Cer. However, metabolism of Cer does not appear to explain the failure of bSMase in inducing apoptosis. Therefore our data support the hypothesis that resistance to apoptosis is not a result of metabolic removal of Cer.

MATERIALS AND METHODS

Materials

Cell culture plasticware was from Costar (Cambridge MA, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), PBS and Hanks balanced salt solution (HBSS) were from Gibco (Paisley, U.K.) and fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). C₂-Cer and C₂-DH-Cer were obtained from Biomol Research Laboratories (Plymouth Meeting PA, U.S.A.). Sphingosine (So), *Staphylococcus aureus* SMase and alkaline phosphatase-conjugated sheep anti-rabbit IgG were from Sigma Chemical Company (St. Louis MO, U.S.A.). D,L-threo 1-phenyl-2-decanoylamino-3-morpholino-1-propanol

Abbreviations used: bSMase, bacterial sphingomyelinase; Cer, ceramide; DH-Cer, dihydroceramide; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GalCer, galactosylceramide; GlcCer, glucosylceramide; GSL, (glyco)sphingolipids; HBSS, Hanks balanced salt solution; LacCer, lactosylceramide; MAPP, D-erythro-2-N-myristoylamino-1-phenyl-1-propanol; PARP, poly(ADP-ribose) polymerase; PDMP, D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; SM, sphingomyelin; So, sphingosine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

¹ To whom correspondence should be addressed.

(PDMP) was obtained from Matreya (Pleasant Gap PA, U.S.A.) and *D-erythro-2-N-myristoylamino-1-phenyl-1-propanol* (MAPP) was provided by Dr. A. H. Merrill, Jr. (Department of Biochemistry, Emory University, Atlanta GA, U.S.A.). The cell death detection ELISA^{plus} kit, the *in situ* cell death detection kit and the polyclonal anti-poly(ADP-ribose) polymerase (PARP) antibody were from Boehringer Mannheim (Almere, The Netherlands). Immobilon-P membrane was purchased from Millipore Corporation (Bedford MA, U.S.A.). L-[3-³H]serine was purchased from Amersham International (Berk., U.K.) and [³H]acetic anhydride was from ICN Biomedicals (Costa Mesa CA, U.S.A.). En³Hance spray was from Dupont (Brussels, Belgium).

Cell culture and experimental conditions

The human colon adenocarcinoma cell line HT29^{rev} [17] was cultured in DMEM containing 25 mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) heat-inactivated (56 °C, 30 min) FCS. Cells were seeded at a density of 4×10^4 cells/cm² and maintained in a water-saturated atmosphere of 5% CO₂/95% air at 37 °C. Medium was refreshed every 48 h and the culture was passed weekly by trypsinization. All treatments took place during the exponential growth phase and in the presence of 10% FCS, unless stated otherwise. Lipids and MAPP were delivered to the cells by ethanolic injection (the final ethanol concentration never exceeded 0.25%), whereas PDMP was added from a pre-warmed stock solution in PBS. bSMase was added directly from the stock as purchased. Cells under control conditions were treated with vehicle alone.

Analysis of DNA fragmentation

Cells were washed twice and medium containing 1% (v/v) FCS and test agents was added. After 24 h, cells were harvested by scraping and washed twice by resuspension in PBS and subsequent centrifugation (5 min, 2000 g). Pelleted cells were lysed in 10 mM Tris/HCl (pH 8.0) buffer containing 0.6% (w/v) SDS and 10 mM EDTA and incubated with 100 µg/ml DNase-free ribonuclease I (1 h, 37 °C). NaCl was added until a final concentration of 1 M. After a 1 h incubation at 4 °C, the lysates were centrifuged (45 min, 12 500 g, 4 °C). The supernatants were extracted with phenol/chloroform and chloroform respectively. DNA was precipitated overnight in 2.2 vol of ethanol at -20 °C and subsequently centrifuged (30 min, 12 500 g, 4 °C). The pellet was redissolved in 10 mM Tris/HCl (pH 8.0) containing 1 mM EDTA. Aliquots of 25 µg of DNA were resolved by electrophoresis at 70 V for 90 min on 1.5% agarose gels impregnated with 0.1 µg/ml ethidium bromide. DNA was visualized under UV light and the gel was photographed.

Cytoplasmic histone-associated DNA ELISA

After harvesting and washing of the cells in HBSS, the total amount of protein was determined according to the method of Smith et al. [18], using BSA as standard. Equal amounts of protein were incubated in lysis buffer as provided by the cell death detection ELISA^{plus} kit. Subsequently, nuclei were separated from the cytoplasmic fraction by centrifugation (5 min, 2000 g). The relative enrichment of DNA-histone complexes in the cytoplasmic fraction was measured with the ELISA according to the manufacturer's manual.

In situ cell death detection

Cells were grown on glass coverslips and, after treatment, incubated in 4% (w/v) paraformaldehyde (30 min, 20 °C) for

fixation. After washing the coverslips in HBSS, cells were permeabilized in 0.1% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate (2 min, 4 °C). Subsequently, samples were subjected to the *in situ* fluorescein terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) procedure with the aid of an *in situ* cell death detection kit. Cells were examined using an Olympus AX70 fluorescence microscope. Photographs were taken with an Olympus PM-20 photography unit and Ilford HP5-plus films, which were exposed and processed at 1600 ASA.

Western blotting of PARP

After harvesting and washing of the cells, the total amount of cellular protein was determined following the procedure of Smith et al. [18], using BSA as standard. Subsequently, cells were lysed by sonication (1 min, 20 °C) and subsequent incubation (15 min, 65 °C) in 33 mM Tris/HCl (pH 7.6) buffer containing 33 mM glucose, 7 mM EDTA, 0.7 mM PMSF, 2 M urea, 2% (v/v) 2-mercaptoethanol and 1% (w/v) SDS. Protein (30 µg per lane) was run on SDS/PAGE (10%) minigels following the protocol of Laemmli [19]. Proteins were electrotransferred onto Immobilon-P PVDF membranes using the method of Towbin et al. [20]. The membranes were rinsed with PBS and incubated in blocking buffer consisting of 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.3% (v/v) Tween-20 and 5% (w/v) non-fat dry milk (2 h, 20 °C). For immunoreaction, the membranes were incubated overnight with the primary antibody (anti-PARP, 1:2000) in blocking buffer. Membranes were washed with blocking buffer and incubated with alkaline phosphatase-conjugated sheep anti-rabbit IgG in blocking buffer (2 h, 20 °C). Immunoreactive proteins were detected using *p*-Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates in 100 mM Tris/HCl (pH 9.5) containing 100 mM NaCl and 5 mM MgCl₂. The reaction was terminated by adding 10 mM Tris/HCl (pH 7.5) containing 1 mM EDTA.

Total SM mass measurement

After treatment, cells were harvested and extracted according to the method of Bligh and Dyer [21] and the total amount of phospholipid in the extract was determined as described by Böttcher et al. [22]. Acylglycerolipids were hydrolysed by incubation (1 h, 37 °C) of the extract in CHCl₃/CH₃OH (1:1, by vol.) containing 0.1 M KOH. Remaining lipids were re-extracted and SM was isolated by TLC, using CHCl₃/CH₃OH/CH₃COOH/H₂O (60:30:8:5, by vol.) as mobile phase. SM-containing spots were identified with standards and assessed for phosphate contents.

Radiolabelling and analysis of cellular glycosphingolipids (GSL)

The total pool of GSL was radiolabelled by growing the cells in the presence of 3 µCi/ml L-[3-³H]serine, a precursor molecule for GSL biosynthesis, for 48 h. Four hours before the start of the experiments, cells were washed twice with HBSS and fresh medium, with or without 25 µM PDMP or 25 µM MAPP, was added. After treatment with 0.1 unit/ml bSMase, the medium was removed and cells were fixed in ice-cold CH₃OH. Cells were harvested by scraping and lipids were extracted. In addition to the cells, in some cases also the culture media were extracted and evaluated for the presence of radiolabelled products. Aliquots of the lipid extracts were taken for determination of the total amount of lipid-incorporated radioactivity. Acylglycerolipids were hydrolysed during a 1 h incubation at 37 °C in CHCl₃/CH₃OH (1:1, v/v) containing 0.1 M KOH. The remaining lipids were re-extracted and applied to HPTLC plates. Plates were

developed in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (14:6:1, by vol.) in the first dimension and in $\text{CHCl}_3/\text{CH}_3\text{COOH}$ (9:1, v/v) in the second dimension. Plates were dried and, after staining in I_2 vapour, Cer-containing spots were scraped. Plates were then sprayed with 2.5% (w/v) H_3BO_3 in CH_3OH and developed once more in the second dimension, this time using $\text{CHCl}_3/\text{CH}_3\text{OH}/25\%$ (w/v) NH_4OH (13:7:1, by vol.) as the mobile phase. After spraying with En^3Hance and autoradiography, SM, glucosylceramide (GlcCer)-, galactosylceramide (GalCer)-, lactosylceramide (LacCer)- and So-containing spots were scraped from the plates after identification with standards. All lipids were subjected to scintillation counting.

Ganglioside mass studies

After treatment, cells were treated with trypsin, washed and counted. Gangliosides were extracted as described by Senn et al. [23]. Cells (6×10^7) were subjected to a one-phase extraction in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v). After sonication and centrifugation (5 min, 1000 g), pellets were re-extracted in $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:2, v/v) and supernatants were pooled. After centrifugation (15 min, 3000 g) the supernatant was dried under N_2 . Lipids were dissolved in $\text{C}_6\text{H}_{14}\text{O}/\text{CH}_3(\text{CH}_2)_3\text{OH}$ (3:2, v/v) and NaCl was added to a final concentration of 0.85 mM. After centrifugation (10 min, 2000 g) the aqueous lower phase was washed with $\text{C}_6\text{H}_{14}\text{O}/\text{CH}_3(\text{CH}_2)_3\text{OH}$ (3:2, v/v) and subsequently lyophilized. The samples were dissolved in $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (1:1, v/v), sonicated and loaded onto a Sep-Pak C_{18} cartridge which had been pre-washed with CH_3OH , $\text{CH}_3\text{OH}/\text{CHCl}_3$ (1:1, v/v), CH_3OH and $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (1:1, v/v). After rinsing with H_2O , the cartridge was dried by N_2 . Gangliosides were eluted from the cartridges by successive washes with CH_3OH and $\text{CHCl}_3/\text{CH}_3\text{OH}$ (1:1, v/v) and dried under N_2 . The eluate was applied to HPTLC plates which were developed in $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (50:42:11, by vol.) containing 0.2% (w/v) CaCl_2 . After drying, plates were sprayed with $\text{C}_6\text{H}_5\text{OH}/37\%$ (w/v) HCl (4:1, v/v) containing 300 mg of dimethylaminobenzaldehyde (Ehrlich's spray), heated (180°C , 15 min) and photographed.

Synthesis of and studies with radiolabelled $\text{C}_2\text{-Cer}$

Radiolabelled $\text{C}_2\text{-Cer}$ was prepared as described [24]. Briefly, 100 mM D-erythro-So in CH_3OH , containing 8 mM NaOH, was mixed with an equal volume of 100 mM [^3H]acetic anhydride (specific activity 70 mCi/mmol) in C_6H_6 . The mixture was incubated for 1 h at 37°C and the reaction was stopped by the addition of 10 vol. of 0.2 M NaOH in CH_3OH . Lipids were extracted and dried under N_2 . Radiolabelled $\text{C}_2\text{-Cer}$ was purified on HPTLC plates, impregnated with 2.5% H_3BO_3 in CH_3OH (w/v) and using $\text{CHCl}_3/\text{CH}_3\text{OH}/25\%$ (w/v) NH_4OH (13:7:1, by vol.) as the solvent system. After elution from the silica, radiolabelled $\text{C}_2\text{-Cer}$ was mixed with non-radioactive $\text{C}_2\text{-Cer}$ to a final concentration of 10 mM. The final specific radioactivity was 19 mCi/mmol.

After incubation with $50 \mu\text{M}$ [^3H] $\text{C}_2\text{-Cer}$, cells were washed twice with PBS and harvested by scraping in ice-cold CH_3OH . The washing fluid was pooled with the removed medium and centrifuged (5 min, 1000 g). Samples for scintillation counting were taken from the supernatant. The pellet was pooled with the harvested cells and extracted according to the method of Bligh and Dyer [21]. Samples of the extracts were taken for scintillation counting. The extracts were analysed on HPTLC plates impregnated with H_3BO_3 [2.5% (w/v) in CH_3OH], and the plates were developed in $\text{CHCl}_3/\text{CH}_3\text{OH}/25\%$ (w/v) NH_4OH (13:7:1, by vol.). After spraying with En^3Hance and autoradiography, all spots were scraped and subjected to scintillation counting.

RESULTS AND DISCUSSION

bSMase treatment fails to induce apoptosis in HT29 cells, in contrast to $\text{C}_2\text{-Cer}$

Previous studies have shown that elevated Cer, as generated by exogenous addition of bSMase, induces apoptosis in a number of cell types [2,5]. However, when HT29^{rev} cells were incubated with 0.1 unit/ml bSMase for 24 h, apoptosis did not occur. This was revealed by the absence of characteristic DNA fragmentation, as assayed by gel electrophoresis (Figure 1), and an ELISA detecting

Figure 1 Assessment of apoptotic DNA fragmentation by gel electrophoresis

Semi-confluent cultures of HT29^{rev} cells were exposed to $10 \mu\text{M}$ (lane B), $30 \mu\text{M}$ (lane C) or $50 \mu\text{M}$ $\text{C}_2\text{-Cer}$ (lane D), $50 \mu\text{M}$ $\text{C}_2\text{-DH-Cer}$ (lane E), 0.1 unit/ml bSMase (lane F) or 0.1 unit/ml bSMase in combination with $25 \mu\text{M}$ PDMP (lane G). After 24 h, DNA was extracted and resolved by gel electrophoresis. A typical gel photographed under UV light is shown. Lane A shows an untreated control.

Figure 2 Apoptosis detection by cytoplasmic histone-associated DNA ELISA

HT29^{rev} cells were treated with bSMase (A), $\text{C}_2\text{-Cer}$ (B) or $\text{C}_2\text{-DH-Cer}$ (D) at the indicated concentrations for 24 h. Cells represented by (C) were treated with $50 \mu\text{M}$ $\text{C}_2\text{-Cer}$ for the indicated time periods. After treatment, cytoplasmic fractions were separated from nuclear fractions and samples were subjected to an ELISA that detected DNA-histone complexes. Data are expressed relative to untreated control cells.

Figure 3 *In situ* apoptosis detection by TUNEL

After treatment with 0.1 unit/ml bSMase (A), 50 μ M C₂-Cer (B) or 50 μ M C₂-DH-Cer (C) for 24 h, HT29^{rev} cells were fixed and permeabilized. Subsequently, cells were subjected to TUNEL using fluorescently labelled nucleotides as substrate. The specimens were then examined by fluorescence microscopy and photographed. (D) shows untreated control cells. (Scale bar = 10 μ m)

cytoplasmic histone-associated DNA fragments (Figure 2A) [25]. Furthermore, no labelling was observed when bSMase-treated cells were subjected to an *in situ* TUNEL reaction, using fluorescent nucleotides as substrate (Figure 3A) [26]. The assays described above are all based on fragmentation of DNA as a marker for the apoptotic process. An alternative method for apoptosis studies is based on detection of the cleavage of PARP by immunoblotting. PARP, a 113 kDa protein involved in DNA repair processes [27], is a substrate for specific proteases belonging to the interleukin-1 β -converting enzyme family, such as apopain and Nedd-2 [28,29], which become active during the early stages of apoptosis. The use of a polyclonal antibody, which detects both intact PARP as well as its 89 kDa and 24 kDa cleavage

Figure 4 Immunodetection of PARP and PARP fragments by Western blot analysis

Semi-confluent cultures of HT29^{rev} cells were treated for 24 h with 50 μ M C₂-Cer (B), 50 μ M C₂-DH-Cer (C), 0.1 unit/ml bSMase (D), 0.1 unit/ml bSMase in combination with 25 μ M PDMP (E), 0.1 unit/ml bSMase in combination with 25 μ M MAPP (F), or 0.1 unit/ml bSMase in combination with both 25 μ M PDMP and 25 μ M MAPP (G). Cell lysates were subjected to SDS/PAGE followed by Western blotting. The polyclonal antibody against PARP immunoreacts with 113 kDa intact PARP and with its 89 kDa and 24 kDa cleavage products. Lane A shows untreated control cells.

Table 1 Activity control of exogenously added bSMase

HT29^{rev} cells were cultured in the presence of 0.1 unit/ml bSMase. At the indicated time points, total lipids were extracted and analysed for SM mass. Data are the average values of two independent experiments, expressed as percentages compared with untreated controls. The last row shows the relative SM content of cells which were incubated for 2 h in medium taken from cells that had been treated for 48 h.

Time (h)	Control	0.1 unit/ml bSMase (% of control)
0	100.0	104.1
2	100.0	22.5
48	100.0	11.6
48 + 2	100.0	25.3

products, allowed us to monitor the apoptotic process. Lysates of bSMase-treated cells contained only intact PARP (Figure 4). The PARP-based assay thus confirmed the observations obtained with the DNA-degradation detection techniques. Incubations with bSMase concentrations higher than 0.1 unit/ml (Figure 2A) and during time intervals beyond 48 h were equally ineffective in apoptosis induction. In addition to the absence of apoptosis induction, no effects of bSMase on growth or morphology of HT29^{rev} cells were observed (results not shown).

The possibility existed that bSMase had lost its activity in the course of the experiment. However, this could be excluded, since control experiments revealed that the enzyme retained its SM-hydrolysing capacity for at least 48 h (Table 1). When the cellular content of endogenous SM was measured in HT29^{rev} cells, a reduction of almost 80% compared with control values was observed after 2 h of treatment. This reduction was stable up to at least 48 h. When incubation medium containing bSMase, which had already been active for 48 h, was transferred to fresh cells, again a drastic reduction in SM content occurred after 2 h of incubation. These data show that bSMase retained its full activity throughout the experiments.

The foregoing results raised the question as to whether HT29^{rev} cells are susceptible at all to Cer-induced apoptosis. Therefore, cells were incubated with C₂-Cer, a short-chain analogue of the

Figure 5 Short- and long-term effects of bSMase on L-[3-³H]serine-labelled GSL pools

Sphingolipid pools were radiolabelled by growing HT29^{rev} cells for 48 h in the presence of L-[3-³H]serine. Thereafter ($t = 0$ min), 0.1 unit/ml of bSMase (●) or 0.1 unit/ml of bSMase in combination with 25 μ M PDMP (○) was added for the indicated time-periods. Total lipids were extracted and subjected to alkaline hydrolysis. Remaining lipids were separated by two-dimensional TLC and the amount of radioactivity in the SM (A), Cer (B), GlcCer (C), GalCer (D), LacCer (E) and So (F) pools was determined. Average values $\pm$ S.D. of three experiments are shown. Data are expressed as d.p.s./10⁵ d.p.s. in the total lipid extracts.

lipid. As shown in Figure 1, C₂-Cer induced DNA fragmentation in a dose-dependent fashion, starting at a concentration of 10 μ M and reaching full activity at 30–50 μ M. In contrast, incubation of HT29^{rev} with C₂-DH-Cer, even at a concentration as high as 50 μ M, did not result in apoptosis. Apparently, apoptosis induction by C₂-Cer is structurally specific, which is in agreement with previous studies [30]. These results were confirmed by the observation that C₂-Cer-treated cells showed a strong, dose-dependent enrichment in cytoplasmic DNA–histone complexes, indicating increased DNA-fragmentation due to apoptosis, whereas C₂-DH-Cer-treated cells showed only a marginal increase (Figures 2B and 2D). C₂-Cer-induced DNA fragmentation turned out to be time-dependent (Figure 2C), starting approximately 8 h after addition of the lipid and reaching plateau values within 20 h of incubation. As shown in Figure 3(B), fragmented nuclei, stained due to the TUNEL reaction, further demonstrated the occurrence of apoptosis [26]. Again, C₂-DH-Cer served as a negative control (Figure 3C). Finally, the lysate of C₂-Cer-treated cells contained 89 kDa and 24 kDa fragments of PARP, whereas C₂-DH-Cer-treated cells only contained intact 113 kDa PARP (Figure 4). This observation

confirms the results obtained by the DNA-fragmentation detection methods. In conclusion, HT29^{rev} cells are susceptible to apoptosis induction by C₂-Cer but do not respond to Cer generated by exogenously added bSMase.

Metabolic processing of bSMase-generated Cer

These results prompted the question as to why Cer, generated in the outer leaflet of the plasma membrane by bSMase, is unable to induce apoptosis, whereas exogenous short-chain C₂-Cer is. An explanation could be that differences exist in the way these two forms of Cer are metabolically processed. For instance, endogenous Cer may be prone to rapid turnover to other GSL before being able to activate the molecular targets involved in propagation of the apoptotic signal.

In order to test this hypothesis, metabolic processing of elevated Cer was measured after radiolabelling of all cellular GSL with L-[3-³H]serine. As shown in Figure 5(A), bSMase started to hydrolyse SM immediately upon addition. Compared with control values, the SM level was reduced to approximately 20% within 2 h and remained low as long as the enzyme was present. Cer, the direct breakdown product of SM, increased 5–6-fold compared with untreated control cells. The rise in Cer levels started immediately upon addition of the enzyme and was maximal after 1–2 h. Despite the continuous presence of active bSMase, Cer levels eventually decreased again to basal values (Figure 5B).

The latter observation indicates that HT29^{rev} cells possess a mechanism by which excessive Cer can be processed effectively. We therefore performed a series of experiments to determine the fate of this Cer pool. When lipids were extracted from the incubation media and analysed for the presence of radiolabelled Cer, only trace amounts were detected (results not shown). Thus, release of Cer into the incubation medium did not significantly contribute to the decrease in Cer levels. When bSMase was added at a concentration of 0.05 unit/ml, the kinetics of SM hydrolysis were identical with those observed when 0.1 unit/ml was used (results not shown). This shows that the amount of enzyme present in the culture medium was not rate-limiting and it is therefore reasonable to assume that resynthesis of SM from Cer will be followed by hydrolysis. Hence, this metabolic pathway will not play a significant role with respect to turnover of the excessive Cer pool. Instead, excessive Cer was most likely used as a substrate for cerebroside and complex ganglioside synthesis. As shown in Figures 5(C) and 5(D), the levels of both GlcCer and GalCer increased concomitantly with the decrease in Cer, suggesting a metabolic relationship. Quantitatively, however, this increase in cerebroside is not sufficient to account for the total decrease in Cer. GlcCer, being a substrate for the biosynthesis of LacCer and more complex GSL, turned out to be further metabolized. A net increase in both LacCer (Figure 5E) and of the total mass of gangliosides, in particular GM₁ and GM₃, was observed (Figure 6). Additional evidence for shuttling of Cer into this metabolic route was provided by experiments in which cells were co-incubated with 0.1 unit/ml bSMase and 25 μ M PDMP, a potent GlcCer synthase inhibitor [31]. Although not blocked completely, the metabolic processing of bSMase-generated Cer was significantly inhibited by PDMP (Figure 5B), which eventually resulted in a Cer level of $263 \pm 19\%$ ($n = 3$) when compared with cells which had been treated with bSMase only ($100 \pm 15\%$, $n = 3$). Thus the metabolic processing of bSMase-generated Cer, which starts several hours after an apparent maximum in the Cer level has been reached, can be partially blocked by co-incubation with a GlcCer synthase inhibitor, thereby causing a relative accumulation of Cer. Co-incubation with bSMase and 25 μ M

Figure 6 Effect of bSMase on endogenous gangliosides

HT29^{rev} cultures were incubated in the absence (C) or presence (S) of 0.1 unit/ml of bSMase for 48 h. Gangliosides were extracted and separated as described in the Materials and methods section. Typical profiles on an Ehrlich's stained TLC plate are shown. The left lanes show GM₁ and GM₃ standards respectively. The right lane (M) shows a mixture of ganglioside standards: the upper band representing GM₁ and the lower bands mainly consisting of GD_{1A}, GD_{1B} and GT_{1B}.

Figure 7 Uptake kinetics of radiolabelled C₂-Cer

Radiolabelled C₂-Cer was prepared as described in the Materials and methods section and applied to semi-confluent cultures of HT29^{rev} cells at a concentration of 50 μ M. At the indicated time intervals, samples were taken from the medium (○) and the cellular lipid extracts (●) and analysed for their radioactive contents.

Table 2 Co-incubation of bSMase with PDMP or MAPP and apoptosis

Semi-confluent HT29^{rev} cultures were treated as indicated for 24 h. After treatment, the cytoplasmic fractions were separated from the nuclei and samples were subjected to an ELISA that detected DNA–histone complexes. The cytoplasmic enrichment of these complexes is expressed relative to the average value of the untreated control ($n = 2-4$). E.F., enrichment factor.

0.1 unit/ml bSMase	25 μ M PDMP	25 μ M MAPP	50 μ M C ₂ -Cer	50 μ M C ₂ -DH-Cer	E.F. $\pm$ S.D
–	–	–	–	–	1.00 $\pm$ 0.31
+	–	–	–	–	1.17 $\pm$ 0.08
–	+	–	–	–	1.64 $\pm$ 0.27
+	+	–	–	–	2.21 $\pm$ 0.53
–	–	+	–	–	1.61 $\pm$ 0.32
+	–	+	–	–	2.23 $\pm$ 0.43
–	+	+	–	–	3.02
+	+	+	–	–	3.10
–	–	–	+	–	11.77 $\pm$ 0.60
–	–	–	–	+	2.56 $\pm$ 1.11

MAPP, an inhibitor of ceramidase [32], resulted in a less pronounced accumulation of Cer ($155 \pm 7\%$, $n = 3$). Apparently, degradation of Cer via So did not significantly contribute to its metabolic removal. This was confirmed by the observation that, in absolute terms, only a limited increase in So levels could be observed upon the addition of bSMase (Figure 5F). In summary, elevated Cer is mainly turned over to cerebroside and complex GSL, while a minor fraction is degraded via So.

C₂-Cer accumulates in the cell

In order to assess whether exogenously added C₂-Cer was subjected to metabolic processing, cells were incubated with [³H]C₂-Cer. As shown in Figure 7, uptake of C₂-Cer started immediately upon addition and 50% of the maximal uptake was reached within 10 min. As revealed by TLC analysis, cell-associated C₂-Cer remained metabolically largely inert. Even after an overnight incubation, only trace amounts of C₂-SM (1.5%) and C₂-GlcCer (1.4%) could be detected. Thus, C₂-Cer accumulated in the cells, without substantial metabolic turnover.

Studies performed with fluorescent lipid derivatives in artificial membrane systems clearly show that short-chain lipids easily undergo both inter- and trans-bilayer transfer [33,34]. When added to cells, these lipids accumulate at various intracellular sites [35]. The extent of this mobility is strongly dependent on the acyl chain length [36]. In conjunction with this, it is reasonable to assume that, when added to cells, C₂-lipid analogues easily cross bilayers and freely diffuse through the cell. This in contrast to, for instance, C₁₂-lipids or natural lipids. Therefore C₂-Cer accumulation most likely occurred at intracellular sites.

Metabolic removal of bSMase-generated Cer does not explain its inability to induce apoptosis

In contrast to an accumulation of C₂-Cer, the Cer pool produced upon bSMase treatment increased only transiently. Excess Cer was routed into the biosynthetic pathway to cerebroside and complex glycolipids. This suggests that the difference in metabolic fate could explain the difference in apoptotic induction capacity. However, we consider this possibility unlikely for the following reasons. (1) The kinetics of removal of bSMase-generated Cer are slow compared with the kinetics of onset of apoptosis. C₂-Cer-induced apoptosis is detectable 8–12 h after addition of the lipid (Figure 2C), and the actual onset of this process must have occurred even earlier. Within this time interval bSMase-generated Cer reached its peak value, whereas it took at least 24 h before

all excessive Cer was metabolically processed by the cell (Figure 5B). Thus, the kinetics of changes in the Cer pool induced by bSMase treatment are such that apoptosis could have occurred. (2) Even when the down-regulation of the abnormally high cellular Cer level induced by bSMase treatment was counteracted by co-incubation with PDMP, MAPP, or both, substantial apoptosis did not occur (Figure 1 and Table 2). Although some DNA fragmentation could be detected, this was marginal when compared with the extent of apoptosis induced by C₂-Cer (Figure 2B and Table 2). In addition, PARP-cleavage products were completely absent in cells treated with bSMase in combination with one or both inhibitors (Figure 4). In conclusion, a factor other than metabolic removal prevented induction of apoptosis by bSMase-generated Cer.

An alternative explanation for the absence of apoptotic induction by bSMase-generated Cer could be the absence of transbilayer movement of Cer. This would be a requirement for reaching a cytoplasmic target, since Cer is generated in the exoplasmic leaflet of the plasma membrane, which is topologically equivalent to the luminal leaflet of intracellular organelles. From this point of view, Cer would, even after endocytic uptake, remain associated with compartments distinct from the cytosol, and might not come into contact with target enzymes involved in the propagation of the apoptotic signal. In addition to transbilayer movement of Cer, subsequent molecular interaction with the target (enzyme) requires transport of Cer or, alternatively, recruitment of the target to the Cer-containing membrane. It has been assumed that natural Cer undergoes spontaneous flip-flop with a $t_{1/2}$ of only seconds [37]. In analogy to diacylglycerol, which, to some extent, shows structural similarity to Cer, the absence of a polar headgroup will indeed greatly facilitate the traversal of the hydrophobic bilayer interior [38]. Furthermore, the fact that Cer is uncharged will also ease the translocation process. Despite these theoretical considerations, however, no experimental data concerning flip-flop characteristics of natural Cer are available. In addition, no data exist with respect to cytosolic transport mechanisms of Cer generated in the plasma membrane, or in membranes of endocytic organelles. Therefore, the possibility remains that the absence of Cer translocation in the plasma membrane, or subsequent cytosolic transport, prevents bSMase from inducing apoptosis. In this respect, it has recently been described that molt-4 cells, which had been transfected with bSMase, undergo apoptosis upon promotor induction [39]. In contrast, when these cells were exposed to exogenous bSMase, apoptosis did not occur. This suggests that a specific, non-exoplasmic pool of SM is involved in apoptosis-related signal transduction. However, in contrast to Molt-4 and HT29^{rev}, other cell types, such as the U937 and Jurkat-T, are known to be susceptible to exogenous bSMase treatment, in terms of apoptosis [2,5]. Therefore, the question arises as to whether these cells do express Cer translocation activity, in contrast to HT29^{rev} and Molt-4 cells. Future research will aim at elucidation of the role of transbilayer movement of Cer in its apoptosis-inducing properties.

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