The role of ceramide in apoptosis

Tepper, A.D.

Publication date
2000

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: https://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Glucosylceramide synthase does not attenuate the ceramide pool accumulating during apoptosis induced by CD95 or anti-cancer regimens.

Annemiek D. Tepper
Sander H. Diks
Wim J. van Blitterswijk
Jannie Borst

The Journal of Biological Chemistry, in press
Glucosylceramide synthase does not attenuate the ceramide pool accumulating during apoptosis induced by CD95 or anti-cancer regimens.

Annemiek D. Tepper, Sander H. Diks, Wim J. van Blitterswijk and Jannie Borst
From the Division of Cellular Biochemistry, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

The abbreviations used are: Cer, ceramide; Eto, etoposide; GalCer, galactosylceramide; GCS, glucosylceramide synthase; GlcCer, glucosylceramide; NBD, N-[6-[[7-nitro-benz-2-oxa-1,3-diazol-4-yl]amino]caproyl]; NBD-Cer, N-(C_6-NBD)-sphingosine; NBD-SM, C_6-NBD-sphingosylphosphorylcholine; PC, phosphatidylcholine; PS, phosphatidylserine; SM, sphingomyelin, SMase, sphingomyelinase.

SUMMARY

Ceramide (Cer) accumulating during the execution phase of apoptosis is generated from plasma membrane sphingomyelin (SM), which gains access to a sphingomyelinase due to phospholipid scrambling (Tepper, A.D., Ruurs, P., Wiedmer, T., Sims, P., Borst, J., and van Blitterswijk, W.J. (2000) J. Cell Biol. 150, 155-164). To evaluate the functional significance of this Cer pool, we aimed to convert it to glucosylceramide (GlcCer), by constitutive overexpression of glucosylceramide synthase (GCS). Jurkat cells, retrovirally transduced with GCS cDNA, showed a 10-12-fold increase in GCS activity in vitro and a 7-fold elevated basal GlcCer level in vivo. However, Cer accumulating during apoptosis induced by ligation of the death receptor CD95, treatment with the anti-cancer drug etoposide or exposure to γ-radiation was not glycosylated by GCS. Likewise, Cer liberated at the plasma membrane by bacterial SMase was not converted by the enzyme. Thus, GCS, located at the Golgi complex, is topologically segregated from Cer produced in the plasma membrane. In contrast, de novo synthesized Cer as well as an exogenously supplied cell-permeable Cer analog were efficiently glycosylated, apparently due to different Cer topology and distinct physico-chemical behaviour of the synthetic Cer species, respectively. Exogenous cell-permeable Cer species, despite their conversion by GCS, effectively induced apoptosis. We also observed that GCS activity is downregulated in cells undergoing apoptosis. In conclusion, GCS can convert de novo synthesized Cer but not SM-derived Cer and, therefore, the ability of GCS overexpression to protect cells from possible detrimental effects of Cer accumulation are limited.
CHAPTER 8

INTRODUCTION

Accumulation of the sphingolipid ceramide (Cer) is a general phenomenon in cells undergoing apoptosis. Different kinetics and mechanisms of Cer formation have been reported, which may be related to the different stimuli and cell types used. In certain cases, Cer was found to be produced in a biphasic manner, whereas other studies only document a late and sustained Cer response during the execution phase of apoptosis (1-3). A role for Cer in apoptosis induction is suggested in many studies, but this concept has recently been challenged (4). Cer can result from sphingomyelin (SM) hydrolysis, catalyzed by neutral or acid sphingomyelinase (SMase) activities. Both SMase activities have been implicated in Cer production upon apoptotic stimuli including TNFα, CD95, anti-cancer drugs and γ-radiation (5-9). Cer can also be synthesized de novo in the ER, starting with the condensation of serine and palmitoyl-CoA (10). Increased de novo synthesis was reported to be responsible for Cer production during daunorubicin- and etoposide- induced apoptosis (11, 12).

We have documented that Cer accumulation in Jurkat cells in response to CD95 ligation, etoposide treatment or γ-radiation is a consequence of inducer caspase activation and effectively takes place during the execution phase of apoptosis (13). Our most recent work (14) revealed the mechanism underlying this late Cer response: SM residing in the outer leaflet of the plasma membrane gains access to an intracellular neutral SMase as a consequence of lipid scrambling, a universal hallmark of the apoptotic execution phase. We have also provided evidence that the relevance of this SM to Cer conversion lies in the depletion of the plasma membrane of SM. SM is an important structural component of the plasma membrane, contributing to its rigidity by interaction with cholesterol. Failure to hydrolyse SM was accompanied by lack of membrane blebbing, whereas replenishment of SM prevented the apoptotic blebbing process.

Although our previous work provides no evidence for Cer as potential second messenger in the apoptotic process, we sought to unveil such a role of Cer by attenuating its levels. Since the SMase responsible for Cer formation in our system has not been molecularly defined, abrogation of Cer production can as yet not be achieved by mutation of this enzyme. However, cellular Cer levels are regulated by enzymatic conversion to other sphingolipids, which provides a tool for intervention. Cer can be (re-)utilized for SM synthesis, degraded to sphingosine or further metabolized to glucosylceramide (GlcCer) or galactosylceramide. GlcCer synthesis is catalyzed by glucosylceramide synthase (GCS) (15), a resident type III integral membrane protein on the cytosolic side of the cis/medial Golgi membrane (16, 17). After its translocation to the Golgi lumen by a yet undefined mechanism, GlcCer can be further metabolized to higher glycosphingolipids including GM3 and GD3 gangliosides (18).

It was previously suggested that glycosylation of Cer can protect cells from cancer drug-induced apoptosis. Accumulation of GlcCer was observed in multidrug-resistant (MDR) tumor cells (19), and overexpression of GCS in MCF-7 breast cancer cells conferred resistance to adriamycin and TNFα (20,21). These findings would support the idea that glycosylation of Cer attenuates its capacity to act as a second messenger.
In this paper, we report that constitutive overexpression of GCS by retroviral transduction of the cDNA into Jurkat cells greatly enhanced glycosylation of de novo synthesized Cer and exogenously added short chain Cer species. However, the Cer pool generated from plasma membrane SM in response to anti-cancer regimens, CD95 ligation or treatment with bacterial SMase was not accessible to the enzyme. This finding underscores that the intracellular site of Cer production is a key factor in determining its further metabolism and therewith its functional potential. In addition, it was observed that GCS activity is downregulated during the apoptotic process. Together, these factors limit the possibility to attenuate the Cer response to apoptotic stimuli by increased CGS activity.

**EXPERIMENTAL PROCEDURES**

**Materials** - DOTAP transfection reagent was obtained from Boehringer Mannheim. Zeocin was from Kyla. L-[3-14C]serine (54.0 mCi/mmol), and the enhanced chemiluminescence system (ECL) were purchased from Amersham Pharmacia Biotech. C6-Cer and C2-Cer were obtained from BIOMOL Research Labs Inc. N[6- {7-nitro-benz-2-oxa-1,3-diazol-4-ylamino}caproyl] (NBD)-Cer and C6-NBD-SM were from Molecular Probes. NBD-GalCer was kindly provided by H. Spong (Dept. of Cell Biology and Histology, Academic Medical Center, Amsterdam). Etoposide, UDP-glucose and Bacillus cereus SMase were from Sigma. The anti-CDS95 monoclonal antibody 7C11 was purchased from Immunotech (Marseille, France).

**Construction of Expression Vector** – The cDNA encoding human GCS was digested from pCG-2 (kindly provided by Drs. S. Ichikawa and Y. Hirabayashi, RIKEN, Japan) (15) and subcloned into the EcoRI and SnaBl sites of the retroviral vector LZRS-MS-ires-Zeo/pBR (kindly provided by Dr. F. Michiels, The Netherlands Cancer Institute). This vector is a derivative of LZRS-pBMN-lacZ, which was provided by Dr. G. Nolan (Stanford University School of Medicine).

**Cell Culture and Retroviral Gene Transduction** – The Jurkat clone J16, which has been selected for sensitivity to CD95 stimulation and T cell antigen receptor expression, was cultured as described (22). LZRS-GCS-ires-Zeo/pBR or empty vector were transfected into the 293T cell derived packaging cell line 8BNX-Ampho (kindly provided by Dr. G.P. Nolan) by calcium phosphate precipitation (23). Virus-containing supernatant was harvested 48 h after transfection and stored at -80°C until further use. Jurkat cells were transduced at a density of 0.5 x 10^6 per ml of virus-containing supernatant in the presence of 10 μg/ml DOTAP. Supernatants were removed after overnight incubation and cells were cultured in fresh medium. Selection of transduced cells was initiated after 24 h by the addition of 200 μg/ml Zeocin. The selection process was completed within 2 weeks. Transduced cells were further cultured in the presence of 200 μg/ml Zeocin.

**Glycosylceramide Synthase Assay** - GCS activity of transduced Jurkat cells was determined in vitro and in vivo, based on conversion of the fluorescent cell permeable Cer analog C6-NBD-Cer-C6-NBD-GlcCer. For the in vitro assay, cells were resuspended in homogenisation buffer (20 mM HEPES pH 7.2, 120 mM K+glutamate, 15 mM KCI, 5 mM NaCl, 0.8 mM CaCl2, 2 mM MgCl2 and 1.6 mM EGTA) were lysed by sonication (Branson; 6 bursts at level 4). C6-NBD-Cer (5 nmol per sample) was dried under nitrogen, dissolved in 10 μl ethanol and added to homogenisation buffer containing 5 mM MnCl2, 1 mM UDP-glucose and 0.3% fatty-acid free BSA. Lysate containing 50 μg of protein (determined by BioRad protein assay) was added to this reaction mixture. After incubation at 37°C, lipids were extracted and separated by TLC (chloroform/methanol/25% NH4OH; 70:30:5 v/v). NBD-lipids were visualized by UV illumination. In
some cases, lipids were scraped and extracted from the silica with 2 ml of chloroform/methanol/HAc (1:2:1 v/v). Silica was spun down and the extracted NBD-fluorescence was measured in a fluorimeter (excitation 470 nm; emission 535 nm). A standard curve was obtained with C₆-NBD-Cer. For the in vivo GCS assay, 5 x 10⁶ cells were resuspended in Hanks' medium buffered with 10 mM HEPES pH 7.4 containing 5 μM C₆-NBD-Cer (added in Hanks' medium containing 1% BSA). Under these conditions, C₆-NBD-Cer is converted to both C₆-NBD-GlcCer and C₆-NBD-SM. After incubation, lipids were extracted and NBD-lipids were analyzed as described above. To monitor the fate of endogenously produced C₆-NBD-Cer during apoptosis, cells (2.5 x 10⁶) were incubated with 4 μM C₆-NBD-SM (in Hepes-buffered Hanks' medium) 10 min prior to apoptosis induction by anti-CD95 antibody (7C11; 200 ng/ml). To determine the in vivo GCS activity during apoptosis induction, cells were exposed to anti-CD95 antibody, etoposide or γ-radiation, bacterial SMase, or left untreated. C₆-NBD-Cer was added (5 μM final concentration) to the cell suspensions 30 min prior to harvest and lipids were extracted and analyzed as described above.

Sphingolipid Quantification - Intracellular Cer, GlcCer and SM levels were determined following metabolic labeling with [³⁴C]-serine as described previously (22, 24). Total lipid extracts were separated by TLC and lipid spots were quantitated by PhosphorImaging. Sphingolipid levels were expressed relative to the radioactivity incorporated into phosphatidylserine plus phosphatidylethanolamine, which remained unaltered upon gene transduction or stimulation of the cells.

Apoptosis Assay - For apoptosis measurements, cells were seeded at 1 x 10⁶ cells/ml, 200 μl/well in round bottom 96 well microtiter plates in serum-free Yssel's medium (25) and incubated for at least 6 h prior to apoptosis induction. Cells were lysed in 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide (26). Fluorescence intensity of propidium iodide-stained DNA was determined in 5000 cells on a FACScan (Becton Dickinson, San Jose, CA) and data were analyzed using Lysys software. Fragmented, apoptotic nuclei are recognized in this assay by their subdiploid DNA content.

RESULTS

Overexpression of Glucosylceramide Synthase

Transduction of Jurkat T cells (J16) with a retroviral vector containing the human GCS cDNA or empty vector yielded the stable cell lines J16-Zeo-GCS and J16-Zeo, respectively. Utilizing the fluorescent Cer analog C₆-NBD-Cer and UDP-glucose, we analyzed whether gene transduction resulted in enhanced GCS activity. In vitro C₆-NBD-GlcCer production in cell lysate of J16-Zeo-GCS was dramatically elevated, being 15-fold higher than in lysates of parental J16 cells or J16-Zeo vector control cells (Fig. 1A, 1B). When the cell-permeable C₆-NBD-Cer was presented to intact cells, J16-Zeo-GCS exhibited a 2.8-3.0-fold increase in vivo GCS activity compared to control cells (Fig. 2A, 2B). Under these conditions, C₆-NBD-Cer was also converted to C₆-NBD-SM and C₆-NBD-GalCer (Fig. 2A). These results clearly demonstrate that J16-Zeo-GCS cells overexpress a catalytically active GCS.
FIG. 1. Effect of GCS transduction on in vitro GCS activity. C₆-NBD-GlcCer production in parental (J16), vector-transduced (J16-Zeo) or GCS-transduced (J16-Zeo-GCS) Jurkat cells was determined using C₆-NBD-Cer as a substrate. Panel A, NBD-lipid analysis by TLC. Sonicated cell lysates were incubated for the time periods indicated with C₆-NBD-Cer and UDP-glucose in the presence of 0.15% fatty acid free BSA. C₆-NBD-GlcCer synthesis was analyzed by TLC. Panel B, Quantitation of in vitro GCS activity. Lipids corresponding to C₆-NBD-GlcCer were scraped off the TLC plates shown in panel A, extracted from the silica and the NBD-fluorescence was measured in a fluorimeter using C₆-NBD-Cer as a standard.
FIG. 2. Effect of GCS transduction on in vivo GCS activity. Intact parental (J16), vector-transduced (J16-Zeo) or GCS-transduced (J16-Zeo-GCS) Jurkat cells were incubated for the time periods indicated in serum-free Hanks’ Hepes medium containing 5 μM C₆-NBD-Cer. **Panel A**, NBD-lipid analysis by TLC. Total lipid extracts were separated by TLC and NBD-lipids were visualized under UV light. C₆-NBD-Cer is predominantly metabolized to C₆-NBD-GlcCer and C₆-NBD-SM and to a minor extent to C₆-NBD-GalCer, as was verified using lipid standards. This is a representative result of four independent experiments. **Panel B**, Quantitation of in vivo GCS activity. Lipid spots corresponding to C₆-NBD-GlcCer were scraped off the TLC plates shown in panel A. Lipids were extracted from the silica and the NBD-fluorescence was measured in a fluorimeter using C₆-NBD-Cer as a standard.
Effect of GCS Overexpression on Steady State Sphingolipid Levels

Next, we analyzed the effect of GCS overexpression on steady state levels of Cer, GlcCer and SM. For this, J16-Zeo and J16-Zeo-GCS cells were metabolically labeled with $[^{14}C]$-serine, which incorporates into the sphingoid backbone of all sphingolipids. A comparison between the relative amounts of radiolabeled lipids in the two cell lines revealed striking differences: In J16-Zeo-GCS cells, basal GlcCer was 7-fold increased, basal SM levels were decreased by ~30%, whereas basal Cer was elevated to ~170% of control values (Fig. 3). We confirmed by lipid charring that the differences observed represent changes in mass rather than altered incorporation of $[^{14}C]$-serine into the lipids (data not shown).

FIG. 3. GCS overexpression alters basal Cer, GlcCer and SM levels. Vector- (J16-Zeo) and GCS-(J16-Zeo-GCS) transduced Jurkat cells were labeled until equilibrium with $[^{14}C]$-serine, followed by extraction and separation of total lipids by TLC. Radiolabeled lipids were quantitated by PhosphorImaging. Data shown represent the mean ± SD from 3 separate experiments in duplicate.

Effect of GCS Overexpression on Cer Accumulation During Apoptosis

To investigate whether GCS overexpression could attenuate the Cer response to apoptotic stimuli, radiolabeled J16-Zeo and J16-Zeo-GCS cells were induced to undergo apoptosis by CD95 ligation or exposure to the DNA-damaging regimens etoposide or $\gamma$-radiation. Regardless of the stimulus used, Cer accumulated in GCS-overexpressing cells to the same extent as in vector control cells (Fig 4, 5AB). Although basal GlcCer was dramatically elevated in J16-Zeo-GCS cells (Fig. 3), apoptotic stimuli did not increase this GlcCer level any further, despite a 3- to 4-fold elevation of Cer.
Cer production and glycosylation during apoptosis. TLC separation of $[^{14}C]$-serine-labeled J16-Zeo and J16-Zeo-GCS cells that were induced to undergo apoptosis by CD95 ligation ($\alpha$CD95; 200 ng/ml mAb 7C11), exposure to etoposide (5 μg/ml) or γ-radiation (γ-IR; 30 Gy), or were left untreated (medium) for the time periods indicated in hours. These stimuli induce the accumulation of Cer (13). In addition, cells were treated with *Bacillus cereus* sphingomyelinase (bSMase, 200 mU/ml) for 15, 30 or 60 min to release Cer at the plasma membrane. Results shown are representative of three independent experiments. SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine.

Quantification of Cer glycosylation during apoptosis. Cer and GlcCer levels in J16-Zeo and J16-Zeo-GCS cells undergoing apoptosis in response to CD95 ligation (Panel A), treatment with etoposide or γ-radiation (Panel B), or exposure to bacterial SMase (Panel C). Cells were labeled with $[^{14}C]$-serine and lipids were resolved by TLC as depicted in Fig. 4. Radioactivity was quantitated by Phosphorimaging and expressed relative to PS plus PE. Panel A, CD95-induced Cer production. Cells were treated with anti-CD95 antibody (200 ng/ml; filled symbols) or left untreated (open symbols) for the time periods indicated. Cer (circles) and GlcCer (squares) levels are the means ± SD of three individual experiments in duplicate. Panel B, DNA damage-induced Cer production. Cells were exposed to etoposide (Eto; 5 μg/ml), γ-radiation (IR; 25 Gy), or left untreated (medium). After 14 h (open bars), 16 h (grey bars) and 18 h (filled bars) of incubation, lipids were extracted. Panel C, Cer released by exogenous SMase. J16-Zeo (open symbols) or J16-Zeo-GCS (filled symbols) cells were exposed to bSMase (200 mU/ml) for the time periods indicated. Cer (circles) and GlcCer (squares) levels were determined and are representative of two independent experiments.
GCS TARGETS de novo SYNTHESIZED BUT NOT SM-DERIVED Cer

A

J16-Zeo

\[ \text{[14C]-lipid} \]

\[ 0.24 \]

\[ 0.18 \]

\[ 0.12 \]

\[ 0.06 \]

2h 4h 6h

J16-Zeo-GCS

\[ \text{[14C]-lipid} \]

\[ 0.24 \]

\[ 0.18 \]

\[ 0.12 \]

\[ 0.06 \]

2h 4h 6h

B

Cer

\[ \text{[14C]-lipid} \]

\[ 0.24 \]

\[ 0.18 \]

\[ 0.12 \]

\[ 0.06 \]

J16-Zeo

medium Eto IR

GlcCer

medium Eto IR

J16-Zeo-GCS

C

\[ \text{[14C]-lipid} \]

\[ 0.4 \]

\[ 0.3 \]

\[ 0.2 \]

\[ 0.1 \]

0 10 20 30 40 50 60

Incubation time (min)

\[ \text{Cer} \]

\[ \text{GlcCer} \]
These findings suggest that overexpressed GCS targets basal Cer but not the pool of Cer which accumulates apoptosis, possibly due to a difference in subcellular localization between agonist-induced Cer and basal Cer. To explore this possibility, we investigated whether the source of Cer accumulation during apoptosis was different from that of basal, i.e. de novo generated, Cer. In our cell system, treatment with 25 μM fumonisin B1 (FB1) during 24 h efficiently blocks de novo Cer synthesis without causing significant cell toxicity (14). We previously showed that CD95 ligation still induces Cer production in FB1-pretreated cells, indicating that it is not a resultant of enhanced de novo Cer synthesis (14). In an analogous experiment we investigated the source of DNA-damage induced Cer. [14C]-serine-labeled J16 cells were treated with FB1 (24 h; 25 μM) to block the de novo pathway and subsequently exposed to etoposide (10 μg/ml) or γ-radiation (20 Gy). After 16 h of incubation in the continued presence of FB1, the Cer content was 3.9 ± 0.5 (etoposide) and 4.2 ± 0.3-fold (γ-radiation) (means ± SD of two independent experiments) above that in FB1-treated unstimulated cells, corresponding to 97% and 93% of the responses observed in the absence of FB1, respectively. Thus, similar to what we found for CD95, Cer formation upon etoposide or γ-radiation is not the resultant of enhanced de novo Cer synthesis. This conclusion is supported by the earlier observation that these DNA-damaging stimuli induce the breakdown of exogenously supplied NBD-SM (14). Taken together, these data strongly suggests that the pool of Cer which accumulates during apoptosis is derived from SM hydrolysis, presumably at the plasma membrane (14), and that this pool is inaccessible to the GCS enzyme, which resides in the Golgi. To verify this interpretation, we examined whether overexpressed GCS convert “excess” Cer, generated artificially at the plasma membrane by exogenous bacterial SMase. SMase-treatment resulted in dramatic Cer accumulation in both J16-Zeo and J16-Zeo-GCS cells (Fig. 4, upper panels; Fig. 5C). However, there was only a slight (but insignificant) increase in GlcCer level upon SMase treatment of GCS overexpressing cells, indicating that this bulk amount of Cer liberated in the plasma membrane does not serve as a substrate for GCS (Fig. 5C). Together, our results clearly demonstrate that GCS does not convert the pool of natural Cer species produced during apoptosis, most likely due to distinct subcellular localization of enzyme (Golgi) and substrate (plasma membrane).

GCS is Inhibited During Apoptosis

Unlike natural Cer generated in the plasma membrane, an exogenously supplied fluorescent cell-permeable Cer analog, C₆-NBD-Cer, is efficiently glycosylated by GCS (Fig. 2). This indicates that such (short-chain) cell-permeable Cer analogs differ from natural Cer species in its capacity to distribute to intracellular membranes. To supplement this data, we assessed whether GCS is able to glycosylate cell-permeable Cer when generated by a SMase in response to apoptotic stimulation. C₆-NBD-SM was added to J16-Zeo-GCS and control cells treated with anti-CD95 antibody and generation of C₆-NBD-Cer species was examined by TLC. In unstimulated cells, C₆-NBD-SM was already converted to C₆-NBD-Cer to some extent, presumably as a result of C₆-NBD-SM endocytosis and accompanying hydrolysis (Fig. 6A). This pool of basal C₆-NBD-Cer was
further metabolized to \( \text{C}_6\text{-NBD-GlcCer} \), which occurred with enhanced efficiency in GCS-transduced cells (Fig. 6A). CD95 stimulation enhanced \( \text{C}_6\text{-NBD-Cer} \) production in a time-dependent manner in both J16-Zeo and J16-Zeo-GCS cells. However, this particular pool of endogenously produced \( \text{C}_6\text{-NBD-Cer} \) did not seem to be further metabolized to \( \text{C}_6\text{-NBD-GlcCer} \). Rather, it appeared that \( \text{C}_6\text{-NBD-GlcCer} \) levels decreased upon CD95 stimulation (Fig. 6A).

Since the in vivo assay had demonstrated that exogenously added \( \text{C}_6\text{-NBD-Cer} \) can reach the Golgi, these results imply that GCS activity is reduced in cells undergoing apoptosis. Indeed, the conversion of exogenously added \( \text{C}_6\text{-NBD-Cer} \) to \( \text{C}_6\text{-NBD-GlcCer} \) was strongly inhibited in a time-dependent manner after apoptosis induction by CD95, etoposide or \( \gamma \)-radiation (Fig. 6B, C). Treatment of cells with bacterial SMase for 2-4 h, which precedes the onset of apoptotic morphological features by at least 6 h did not inhibit GCS activity (Fig. 6C). The in vitro GCS activity was also dramatically reduced in CD95-stimulated cell lysates (Fig. 6D), excluding the possibility that reduced \( \text{C}_6\text{-NBD-GlcCer} \) formation in apoptotic cells is due a reduced accessibility of GCS. We conclude from these experiments that GCS activity is downregulated in cells undergoing apoptosis, irrespective of the stimulus used.

**Apoptosis Sensitivity of GCS-transduced Jurkat Cells**

Although Cer accumulation in the execution phase of apoptosis was unaffected by GCS overexpression, it remained of interest to examine the apoptosis sensitivity of the GCS-overexpressing cells, since GCS might modulate rapid Cer responses, which we have not been able to detect (14, 22), but which have been documented by others (6, 27). Fig. 7A shows that GCS overexpression did not protect cells against CD95-induced apoptosis (nuclear fragmentation). There was even a modest increase in sensitivity to anti-CD95 antibody, but this effect was not statistically significant. In addition, GCS overexpression did not confer resistance to the topoisomerase II inhibitor etoposide or to \( \gamma \)-radiation (Fig. 7B). Since other investigators had found that, in breast cancer cells, GCS can protect against apoptosis induced by Adriamycin (20), another topoisomerase II inhibitor, we tested apoptosis sensitivity to this drug as well. Also in this case, apoptosis was comparable in GCS-transduced cells and control cells (data not shown).

Since we had found that the cell-permeable Cer analog \( \text{C}_2\text{-NBD-Cer} \) was efficiently glycosylated by GCS, we examined whether GCS-transduced Jurkat cells had acquired resistance to apoptosis-induction by (other) synthetic cell-permeable Cer analogs. For comparison, cells were also treated with bacterial SMase, generating natural Cer species in the plasma membrane, that were shown not to be glycosylated by GCS (Fig. 5C). Again, no protective effect of increased GCS expression was observed (Fig. 7C). Rather, GCS-transduced cells displayed increased apoptosis sensitivity to submaximal concentrations of \( \text{C}_2\text{-Cer} \) or \( \text{C}_6\text{-Cer} \).
CHAPTER 8

A

J16-Zeo

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
control & & & \\
\end{tabular}

J16-Zeo-GCS

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
control & & & \\
\end{tabular}

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
NBD-Cer & NBD-GlcCer & NBD-SM & \\
control & & & \\
\end{tabular}

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
\alpha CD95 & & & \\
\end{tabular}

B

J16-Zeo

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
control & & & \\
\end{tabular}

J16-Zeo-GCS

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
control & & & \\
\end{tabular}

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
NBD-Cer & NBD-GlcCer & NBD-SM & \\
control & & & \\
\end{tabular}

\begin{tabular}{cccc}
1 & 2 & 3 & 4 \\
\alpha CD95 & & & \\
\end{tabular}

C

\begin{tabular}{cccc}
14 & 18 & 14 & 18 \\
con & Eto & IR & SMase \\
\end{tabular}

\begin{tabular}{cccc}
14 & 18 & 2 & 4 \\
& & & \\
\end{tabular}

\begin{tabular}{cccc}
\textless NBD-Cer & \textless NBD-GlcCer & \textless NBD-SM & \\
& & & \\
\end{tabular}

D

\begin{tabular}{cccc}
5 & 10 & 20 & \\
control & & & \\
\end{tabular}

\begin{tabular}{cccc}
5 & 10 & 20 & \\
\alpha CD95 & & & \\
\end{tabular}

\begin{tabular}{cccc}
\textless NBD-Cer & \textless NBD-GlcCer & \\
& & & \\
\end{tabular}
DISCUSSION

A Jurkat cell line stably overexpressing GCS was generated with the aim to attenuate Cer accumulation during the execution phase of apoptosis via its enhanced glycosylation to GlcCer. GCS-transduced cells showed increased in vivo and in vitro GCS activity. Basal GlcCer levels in these cells were approximately 7-fold higher than in empty vector-transduced control cells, as evidenced by equilibrium labeling with [\(^{14}\text{C}\)]-serine. Basal SM levels were reduced by about 30% upon GCS-transduction. SM is predominantly synthesized at the luminal side of the cis-Golgi (16, 17), the same cellular compartment where GCS converts Cer to GlcCer and our results strongly suggest that (overexpressed) GCS 'competes' with SM synthase for Cer, their common substrate. The modest increase in basal Cer may reflect an enhanced de novo Cer biosynthesis as a mechanism to compensate for Cer utilization by GCS. In marked contrast to the effects of GCS overexpression on basal Cer/GlcCer metabolism, we found no evidence for enhanced glycosylation of Cer pool accumulating during apoptosis induced by CD95, etoposide or \(\gamma\)-radiation. These findings strongly suggest that the subcellular site of Cer production during apoptosis is segregated from the site of GCS action, the cis-Golgi. This notion is in good agreement with our previous work showing that Cer accumulation in our cell system results from hydrolysis of plasma membrane SM and not from de novo synthesis (14). Moreover, we demonstrate here that the bulk amount of Cer released from plasma membrane SM upon bacterial SMase treatment is virtually not glycosylated by GCS. The rise in Cer upon SMase treatment was maximal after 25-30 min and no significant increase in GlcCer formation was found up to 60 min. In contrast to our results, analysis of the metabolic fate of SMase-generated Cer in HT29 cells indicated that Cer formation (maximal after 1-2 h) was followed by GlcCer production after about 3 h (28). Although the kinetics of Cer production by SMase were much faster in our cell system, it can not be excluded that some Cer is metabolized to GlcCer at later time points. At any rate, GCS overexpression could not prevent the accumulation of Cer.

FIG. 6. GCS activity is inhibited during apoptosis. Panel A, NBD-GlcCer formation from NBD-SM in anti-CD95-treated. J16-Zeo and J16-Zeo-GCS cells were preincubated for 10 min with 4 \(\mu\)M NBD-SM prior to apoptosis induction via CD95 ligation (200 ng/ml 7C11; \(\alpha\)CD95). After the indicated time periods, cells were harvested and lipids extracts were analyzed by TLC. Panel B, NBD-GlcCer formation from NBD-Cer in anti-CD95-treated cells. J16-Zeo and J16-Zeo-GCS cells were stimulated as indicated for Panel A. \(C_6\)-NBD-Cer (5 \(\mu\)M) was added to the cell suspensions 30 min prior to harvest at the time points indicated. Panel C, NBD-GlcCer formation from NBD-Cer in cells treated with other apoptotic stimuli. J16-Zeo-GCS cells suspended in serum-free medium were exposed to etoposide (Eto; 5 \(\mu\)g/ml), \(\gamma\)-radiation (IR; 25 Gy) or were left untreated (con). After 13 h, cells were collected, resuspended in Hepes/Hanks' medium (2.5 \(\times\) 10\(^6\) /ml) and further incubated up to the times indicated. A portion of the untreated cells was exposed to bacterial SMase (SMase; 150 mU/ml) for 2 and 4 h. \(C_6\)-NBD-Cer (5 \(\mu\)M) was added 30 min prior to harvest at the time points indicated. Panel D, in vitro GCS activity of anti-CD95-treated cells. J16-Zeo-GCS cells were left untreated (control) or incubated with anti-CD95 mAb (\(\alpha\)CD95; 200 ng/ml) for 2 h and lysed by sonication. Lysates containing 50 \(\mu\)g of protein were incubated in the presence of 5 \(\mu\)M \(C_6\)-NBD-Cer and UDP-glucose. After the time periods indicated, lipids were extracted and analyzed by TLC to monitor \(C_6\)-NBD-GlcCer formation.
We conclude that the Cer pool that is synthesized de novo in the ER and transported to the Golgi by vesicular trafficking is efficiently targeted by GCS, whereas Cer generated in the plasma membrane is poorly accessible to GCS.

While SMase-liberated Cer was not glycosylated, GCS-transduced Jurkat cells could readily use membrane-inserted C₆-NBD-Cer as a substrate for C₆-NDB-GlcCer synthesis, indicating that this amphipathic Cer analog can reach the Golgi complex. These data illustrate that short-chain Cer analogs do not exactly mimic the behaviour of naturally occurring long-chain Cer species. Due to their solubility in both lipidic and aqueous environments, short-chain Cer analogs that have passed the plasma membrane can apparently reach other intracellular compartments. In contrast, natural Cer species, which contain two long hydrophobic aliphatic chains, strongly favor a lipid environment. The spontaneous exchange rate of long-chain (C₁₆)-Cer between phospholipid vesicles is in the order of days and it has been concluded that, without the aid of an exchange protein, Cer can only interact with molecules located at the same membrane where Cer is formed (29). In contrast, the exchange of C₆-NBD-Cer between lipid vesicles occurs within minutes (30). Other investigators came to essentially the same conclusions, when they observed that natural Cer formed in the lysosomes cannot escape this compartment, in contrast to a fluorescent short-chain Cer analog (31).

Our study also allows the conclusion that GCS activity is inhibited when cells undergo apoptosis. In fact, Cer accumulation is paralleled by a loss of GCS activity. Our results are in agreement with data reported for Kym-1 cells undergoing TNFα-induced apoptosis, in which Cer production (a rapid response peaking after 2 min) was accompanied by decreased in vitro GCS and SM synthase activities (32). However, the failure to glycosylate 'excess' Cer cannot simply be explained by GCS inhibition. Cer released in the plasma membrane upon SMase treatment was not metabolized to GlcCer (Fig. 5C), whereas GCS was still fully active under these conditions (Fig. 6C). Our results suggest that the natural Cer species accumulating during the execution phase of apoptosis are not converted by GCS to GlcCer because i) this pool of Cer is topologically segregated from GCS and ii) the overall GCS activity is downregulated during apoptosis.

Apoptosis sensitivity to CD95, etoposide or γ-radiation was not altered in GCS-transduced cells. GCS overexpression also did not protect against apoptosis induced by cell-permeable C₂-Cer or C₆-Cer. This was unexpected, since (C₆-NBD) Cer was glycosylated by GCS.

---

**FIG. 7. Effect of GCS overexpression on apoptosis sensitivity.** J16-Zeo and J16-Zeo-GCS cells were treated with different apoptotic stimuli and apoptosis was measured by nuclear fragmentation. Panel A, CD95-induced apoptosis. Cells were incubated with anti-CD95 antibody 7C11 at the indicated concentrations, or left untreated (medium). Panel B, DNA damage-induced apoptosis. Cells were treated with etoposide or exposed to γ-radiation (γ-IR) at the doses indicated and apoptosis was read out after 14 h (black bars) or 20 h (grey bars). Panel C, exogenous Cer-induced apoptosis. Cells were incubated in serum-free medium for 15 h in the presence of C₂-Cer, C₆-Cer or B. cereus SMase (bSMase) at the concentrations indicated. Data represent the mean ± SD from three independent experiments.
We did not directly assess it, but other studies have shown that both short-chain Cer species are metabolized upon addition to cells (33), similar to C\textsubscript{6}-NBD-Cer. Thus, despite their glycosylation, these Cer analogs are potent inducers of apoptosis. Paradoxically, dose-response analysis revealed that cells overexpressing GCS even have an increased sensitivity to C\textsubscript{2}-Cer and C\textsubscript{6}-Cer. We should note at this point that the mechanism of short-chain Cer analog-induced apoptosis and particularly the role of GCS therein is not completely understood: Cabot et al. implicated GCS in the protection against cell killing by exogenous Cer (20), suggesting that Cer, and not GlcCer exerts the cytotoxic effect. However, an opposite role for GCS in Cer toxicity is suggested by the work of De Maria et al., who reported that apoptosis induction by Cer requires its conversion to GD3 ganglioside (34). Since the first step in the conversion of Cer to GD3 involves Cer glycosylation to GlcCer, this scenario would imply a sensitizing rather than a protective role for GCS in apoptosis.

The effects of stable GCS overexpression on sphingolipid metabolism and apoptosis sensitivity we observe are markedly different from those reported by Cabot’s laboratory (20, 21, 35). These investigators found increased GlcCer levels in cells displaying multidrug resistance. In MCF-7 breast cancer cells that overexpressed GCS, the Cer pool produced in response to adriamycin or TNF\textalpha was glycosylated and GCS conferred protection against cytotoxicity. The discrepancies between these findings and ours may be explained by different mechanisms and subcellular sites of Cer production involved. These may vary depending on the cell type and stimulus used. We have shown that only de novo generated but not SMase-derived Cer is a substrate for GCS.

The mechanism by which adriamycin causes elevated Cer levels in MCF-7 cells has not been established, but if it stems from de novo synthesis, the effect of GCS can be explained. TNF\textalpha-induced Cer production in MCF-7 cells could either be SM-derived or synthesized de novo, as it ranges from a rapid (10 min) and transient response concomitant with SM hydrolysis (36) to a slow and sustained accumulation starting around 8 h after induction (37). Notably, the suppressive effects of GCS on Cer formation in MCF-7 cells were only seen after a very long time of exposure to adriamycin (24-48h) or TNF\textalpha (48-72h). Alternatively, the explanation for the opposing results data may reside in the different methods employed for either GCS overexpression (a stable polyclonal Jurkat cell population versus inducible MCF-7 clones), lipid quantification (steady-state labeled Jurkat cells versus pulse-labeling of MCF-7 cells at the time of apoptosis induction) and detection of apoptosis (nuclear fragmentation versus cell survival).

Taken together, we have shown that overexpression of GCS in Jurkat cells leads to increased GCS activity and elevated GlcCer levels. These alterations in Cer/GlcCer metabolism have no influence on Cer accumulation associated with the execution phase of apoptosis, most likely due to the distinct subcellular site of Cer production and the GCS enzyme and the inhibition of GCS during apoptosis. Our results emphasize the compartmentalization of SM-derived Cer and Cer glycosylation and demonstrate that the attenuating effect of GCS depends on the mechanism (SMase versus de novo) and subcellular site of Cer production.
GCS TARGETS de novo SYNTHESIZED BUT NOT SM-DERIVED Cer

Acknowledgements

We thank P. Ruurs (Division of Cellular Biochemistry, The Netherlands Cancer Institute) for her expert technical assistance. Drs. S. Ichikawa and Y. Hirabayashi (Laboratory for Glyco-Cell Biology, RIKEN Japan), F. Michiels (Division of Cell Biology, The Netherlands Cancer Institute, Amsterdam) and H. Sprong (Department of Cell Biology and Histology, Academic Medical Center, Amsterdam) are acknowledged for gift of reagents.

REFERENCES