Glucosylceramide Synthase Does Not Attenuate the Ceramide Pool Accumulating during Apoptosis Induced by CD95 or Anti-cancer Regimens

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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, 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 physicochemical behavior 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 down-regulated 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 is limited.

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 biphase 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 endoplasmic reticulum, 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-irradiation 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 hydrolyze 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 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 tumor cells (19),

*This work was supported by Dutch Cancer Society Grant NKI 96-1266. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Cer, ceramide; GalCer, galactosylceramide; GCS, glucosylceramide synthase; GlcCer, glucosylceramide; NBD, N-[6-[7-nitrobenz-2-oxa-1,3-diazol-4-ylamino]caproyl]; NBD-Cer, N-[[NBD-sphingosylphosphorylcholine; SM, sphingomyelin; SMase, sphingomyelinase; TNFα, tumor necrosis factor α; GM3, NeuAcα2,3Galβ1,4Glc-ceramide; GD3, NeuAcα2,8NeuAcα2,3Galβ1,4Glc-ceramide.

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and overexpression of GCS in MCF-7 breast cancer cells conferred resistance to adriamycin and TNFα (20, 21). These findings 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 underlies the fact 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 down-regulated during the apoptotic process. Together, these factors limit the possibility to attenuate the Cer response to apoptotic stimuli by increased GCS activity.

**EXPERIMENTAL PROCEDURES**

**Materials—**N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methy1sulfate transfection reagent was obtained from Roche Molecular Biochemicals (Mannheim, Germany). Jurkat T-cell virus vector, Zeta Pro, was from Kyowa Hakko (Tokyo, Japan). Dickkopf (54.0 mM HEPES, pH 7.2, 120 mM K

**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. 1, A and B). 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 with control cells (Fig. 2, A and B). 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.

**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 [14C]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 3-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 staining that the differences observed represent changes in mass rather than altered incorporation of [14C]serine into the lipids (data not shown).

**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 γ-radiation. Regardless of the stimulus used, Cer accumulated in GCS-overexpressing cells to a much greater extent as in vector control cells (Figs. 4 and 5 (A and B)). 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 3–4-fold elevation of Cer. These findings suggest that overexpressed GCS targets basal Cer but not the pool of Cer, which accumulates during apop-
sis, 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-fold (etoposide) and 4.2 ± 0.3-fold (γ-radiation) (means ± S.D. 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 suggest that the pool of Cer that 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, artificially generated from plasma membrane SM by exogenous bacterial SMase. SMase treatment resulted in dramatic Cer accumulation in both J16-Zeo and J16-Zeo-GCS cells (Figs. 4 (upper panels) and 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 the 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 C₆-NBD-GlcCer, which occurred with enhanced efficiency in GCS-transduced cells (Fig. 6A). CD95 stimulation enhanced C₆-NBD-Cer production in a time-dependent manner in both J16-Zeo and J16-Zeo-GCS cells. However, this particular pool of endogenously produced C₆-NBD-Cer did not seem to be further metabolized to C₆-NBD-GlcCer. Rather, it appeared that C₆-NBD-GlcCer levels decreased upon CD95 stimulation (Fig. 6A). Since the **in vivo** assay had demonstrated that exogenously added C₆-NBD-Cer can reach the Golgi, these results imply that GCS activity is reduced in cells undergoing apoptosis. Indeed, the conversion of C₆-NBD-Cer to C₆-NBD-GlcCer was strongly inhibited when C₆-NBD-Cer was added exogenously to cells undergoing apoptosis induced by CD95, etoposide, or γ-radiation (Fig. 6, B and 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 lysates of CD95-stimulated cells (Fig. 6D), excluding the possibility that reduced C₆-NBD-GlcCer formation in apoptotic cells is due a reduced accessibility of GCS. We conclude from these experiments that GCS activity is down-regulated 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 γ-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. In this case as well, apoptosis was comparable in GCS-transduced cells and control cells (data not shown).

Since we had found that the cell-permeable Cer analog C₆-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 C₃-Cer or C₆-Cer.

**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 [¹⁴C]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 γ-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 cannot 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. We conclude that the Cer pool that is synthesized de novo in the endoplasmic reticulum 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.

Although SMase-liberated Cer was not glycosylated, GCS-transduced Jurkat cells could readily use membrane-inserted C₆-NBD-Cer as a substrate for C₆-NBD-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 behavior 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, 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. 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).

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did not protect against apoptosis induced by cell-permeable C<sub>2</sub>-Cer or C<sub>6</sub>-Cer. This was unexpected, since (C<sub>6</sub>-NBD) Cer was glycosylated by GCS. 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<sub>6</sub>-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<sub>2</sub>-Cer and C<sub>6</sub>-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 of cells from apoptosis induced by cell-permeable Cer analogs.

**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-irradiation (γ-IR) at the doses indicated and apoptosis was read out after 14 h (black bars) or 20 h (gray bars). Panel C, exogenous Cer-induced apoptosis. Cells were incubated in serum-free medium for 15 h in the presence of C<sub>2</sub>-Cer, C<sub>6</sub>-Cer, or B. cereus SMase (bSMase) at the concentrations indicated. Data represent the mean ± S.D. from three independent experiments.
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. (34), who reported that apoptosis induction by Cer requires its conversion to GD3 ganglioside. 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 TNFα 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. TNFα-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 (24–48 h) or TNFα (48–72 h). 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 for either GCS overexpression (a stable polyclonal Jurkat cell and demonstrate that the attenuating effect of GCS depends on inhibition of GCS during apoptosis. Our results emphasize the subcellular site of Cer production and the GCS enzyme and the execution phase of apoptosis, most likely due to the distinct GlcCer levels. These alterations in Cer/GlcCer metabolism have no influence on Cer accumulation associated with the GCS Targets de Novo Synthesized but Not SM-derived Cer

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

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