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Apoptosis induced by N-hexanoylsphingosine in CHP-100 cells associates with accumulation of endogenous ceramide and is potentiated by inhibition of glucocerebroside synthesis

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Abstract

We report that apoptosis induced by N-hexanoylsphingosine (C_6-Cer) in CHP-100 human neuroepithelioma cells associates with accumulation of monohexosylsphingolipids produced not only by short-chain ceramide glycosylation but also through glycosylation of a ceramide pool endogenously produced. By high-performance thin layer chromatography on borate silica gel plates, newly formed monohexosylsphingolipids were identified as glucosylceramides (GluCer); however, accumulation of lactosylceramide or higher-order glycosphingolipids was not observed. GluCer accumulation was fully suppressed by D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; moreover, while this inhibitor had no effect on cell viability when administered alone, it markedly potentiated the apoptotic effect of C_6-Cer. These results provide evidence that activation of GluCer synthesis is an important mechanism through which CHP-100 cells attempt to escape ceramide-induced apoptosis.

Keywords: apoptosis; ceramide; glucosylceramide; CHP-100; neuroepithelioma cells

Abbreviations: C_6-Cer, N-hexanoylsphingosine; Lc-Cer, ceramide with long-chain fatty acid; GluCer, glucosylceramide; HPTLC, high-performance thin-layer chromatography; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol

Introduction

Ceramide (N-acyl-erythro-sphingosine) is now recognized as a key lipid mediator able to trigger the apoptotic programme in various cell systems (Hannun, 1996; Peña *et al*, 1997). Receptor-mediated ceramide generation by sphingomyelinase C activation and apoptosis induction have been reported after cell treatment with various agents, including tumor necrosis factor α (Kim *et al*, 1991; Obeid *et al*, 1993; Jarvis *et al*, 1994), Fas ligand (Trauth *et al*, 1989; Cifone *et al*, 1994) and NGF (Rabizadeh *et al*, 1993; Dobrowsky *et al*, 1994; Casaccia-Bonnefil *et al*, 1996); furthermore, it has been observed that administration of soluble and cell-permeant ceramides can reproduce the apoptotic effects elicited by the above mentioned agents.

Ceramide homeostasis is maintained by mechanisms that control its removal through several potential routes, including deacylation, phosphorylation, glycosylation and sphingomyelin resynthesis (Merril and Jones, 1990; Hannun, 1996). Intriguingly, a body of evidence now indicates that ceramide metabolism may not simply result in lipid downregulation and quenching of its effects, but may also lead to generation of derivatives that provide themselves active mediators in apoptosis induction. Thus, for instance, sphingosine has been demonstrated to induce apoptosis in human leukaemic cells undergoing differentiation by phorbol ester treatment (Otha *et al*, 1995); moreover, sphingosine and N,N,-dimeythylsphingosine have been reported to induce apoptosis in a variety of cancer cell lines more potently than short-chain ceramides (Sweeney *et al*, 1996). The issue of ceramide glycosylation, in the context of apoptosis induction, has recently attracted considerable interest, with evidence provided that activation of this metabolic pathway can either negatively modulate or induce apoptosis. Thus, in breast cancer cells, inhibition of ceramide glycosylation has been shown to reverse a form of resistance to adriamycin, associated with elevated steady-state glucosylceramide (GluCer) levels (Lavie *et al*, 1996; 1997). On the other hand, in human lymphoma cells, GluCer synthase inhibition has been shown to prevent GD3 ganglioside synthesis and apoptosis, as observed after Fas ligation or short-chain ceramide administration (DeMaria *et al*, 1997). We have recently demonstrated that N-hexanoylsphingosine (C_6-Cer) induces apoptosis in CHP-100 human neuroepithelioma cells (Spinedi *et al*, 1998); on the basis of this finding, we were promoted to investigate whether generation of any ceramide-derived metabolite was involved in apoptosis modulation. We report that, in CHP-100 cells, short-chain ceramide elicits endogenous ceramide accumulation; moreover, we provide evidence that both C_6-Cer and endogenously produced ceramide are partly glucosylated during apoptosis induction and that block of this reaction markedly increases programmed cell death.

Results

C_6-Cer induces apoptosis and long-chain ceramide accumulation in CHP-100 cells

We have recently reported that C_6-Cer induces apoptosis in CHP-100 neuroepithelioma cells (Spinedi *et al*, 1998); a time-course of the phenomenon, after cell treatment with 30 μM C_6-Cer, is now shown in Figure 1A. In order to assess whether apoptosis associated with accumulation of any ceramide-derived metabolite, we studied, by high-performance thin-layer chromatography (HPTLC), the changes occurring in cell sphingolipid pattern after C_6-Cer administration. To this aim, samples were developed using two different solvent systems, in order to achieve separation of sphingosine and ceramides (Figure 1B) or neutral glycosphingolipids (Figure 1C). Figure 1B shows that C_6-Cer was rapidly uptaken by CHP-100 cells; moreover, accumulation of ceramide bearing long chain fatty acids (Lc-Cer) was also observed. By fatty acid gas-chromatographic analysis, Lc-Cer mass was estimated less than 3.0 pmol/nmol of lipid phosphate in untreated cells and around 10 pmol/nmol of lipid phosphate after cell treatment for 18 h with 30 μM C_6-Cer. As shown in Figure 2A, C_6-Cer was able to induce radioactivity accumulation in Lc-Cer in cells in which endogenous sphingolipids had been prelabelled with [^{14}C]palmitate; the phenomenon followed a slow kinetics and was associated with decreased sphingomyelin labelling. In keeping with previous reports (Abe *et al*, 1992; Gòmez-Muñoz *et al*, 1995), these results suggest that, in CHP-100 cells, short-chain ceramide is able to induce accumulation of endogenous ceramide.

C_6-Cer and long-chain ceramide undergo metabolism through glycosylation

As shown in Figure 1B, CHP-100 cell exposure to C_6-Cer did not result in sphingosine accumulation, but was consistent with the rapid appearance of a novel product migrating as a single band (lipid Z) and with the marked (albeit slower) accumulation of products migrating as a doublet (lipids XY). Positive staining with α-naphthol indicated that all the newly formed products were glycosphingolipids (not shown); moreover, lipids XY comigrated with cerebroside standards from bovine brain, whereas lipid Z, although being slightly retarded with respect to naturally occurring cerebrosides, migrated faster than lactosylceramide (Figure 1C). Since short-chain ceramides and their derivatives migrate on TLC more slowly than the long-chain counterparts, lipid Z was likely to be a monohexosylsphingolipid derived from direct C_6-Cer glycosylation. In support of this notion we observed the partial release of lipid Z, but not lipids XY, into the culture medium (Figure 2B), a phenomenon indicating defective sphingolipid anchorage to the membrane, due to the presence of short-chain ceramide. In addition, in cells prelabelled with [^{14}C]palmitate and treated with C_6-Cer, radioactivity was found to accumulate in lipids XY but not in lipid Z (Figure 2A), a result clearly indicating that glycosphingolipids migrating as a doublet originate from ceramide endogenously produced whereas the glycosphingolipid migrating as a single band is derived from exogenously administered ceramide.

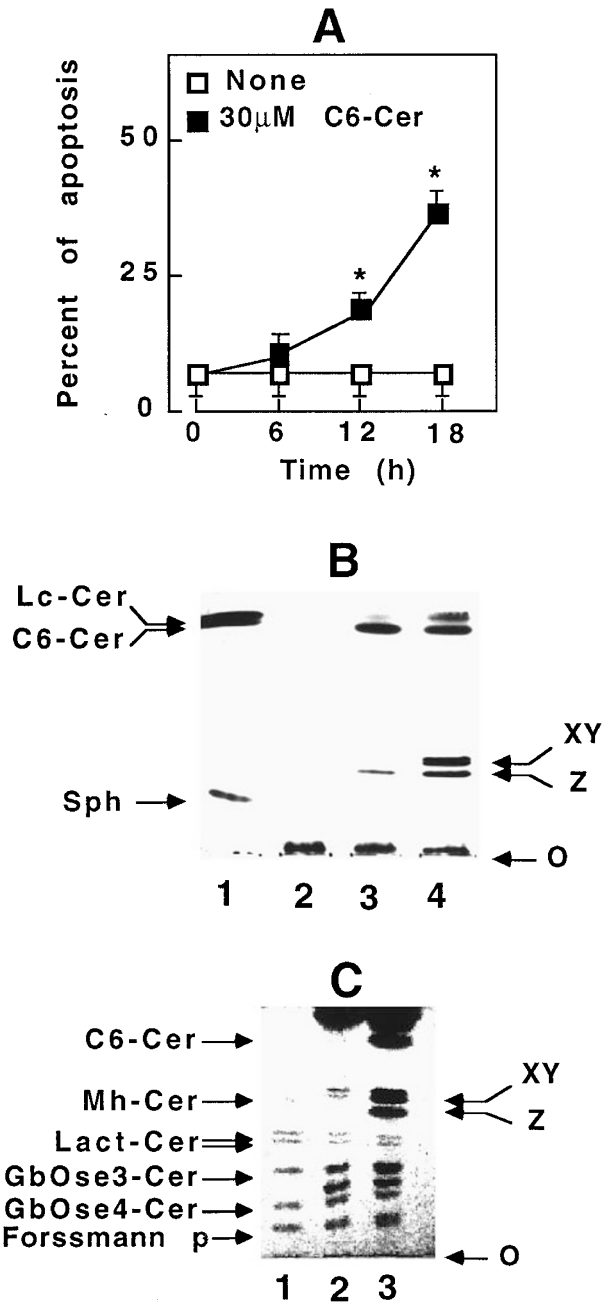

Figure 1 Changes in the sphingolipid pattern in CHP-100 cells undergoing apoptosis after C_6-Cer administration. (**A**) CHP-100 cells were incubated for the indicated times in growth medium containing vehicle (0.1% ethanol) or 30 μM C_6-Cer and apoptosis determined by flow cytometric analysis. Results are means$\pm$S.D. of four different experiments. Statistical significance: $^*P<0.01$, as compared to samples treated with carrier only, as from Student's *t*-test. (**B**) CHP-100 cells were incubated either with vehicle (lane 2) or with 30 μM C_6-Cer for 6 h (lane 3) or 18 h (lane 4). Lipids were extracted by the method of Bligh and Dyer (1959), subjected to mild alkaline hydrolysis and resolved by solvent system I (see text). Lane 1 shows the migration of standards for C_6-Cer, ceramide from bovine brain (Lc-Cer) and sphingosine (Sph). (**C**) CHP-100 cells were incubated either with vehicle (lane 2) or with 30 μM C_6-Cer for 18 h (lane 3). Lipids were extracted by the method of Folch *et al* (1957), subjected to mild alkaline hydrolysis and resolved by solvent system II (see text). Lane 1 shows the migration of standards for C_6-Cer, cerebrosides from bovine brain (Mh-Cer), lactosylceramide (Lact-Cer), globotriaosylceramide (GbOse3-Cer), globotetraosylceramide (GbOse4-Cer) and Forssmann pentasaccharide (Forssmann p). The arrows at the right indicate the origin (O) and the neosynthesized products migrating as a doublet (XY) or single band(Z)

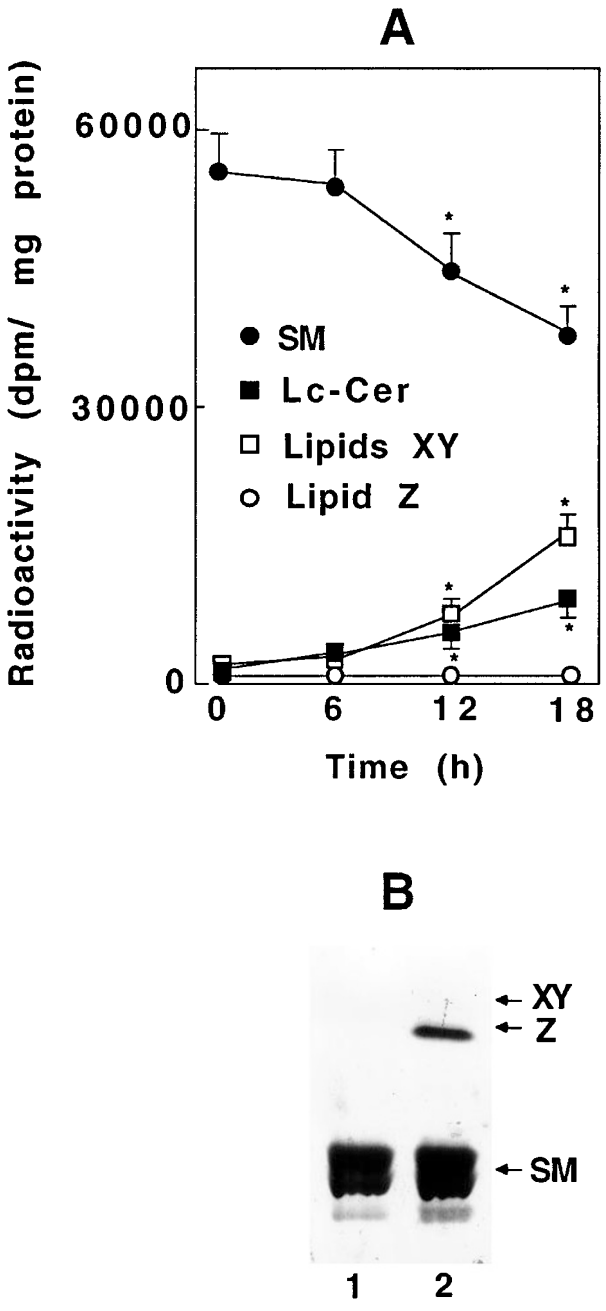

Figure 2 C_6-Cer administration to [^{14}C]palmitate-labelled cells induces radioactivity accumulation in Lc-Cer and in the doublet, whereas the single band is not labelled by [^{14}C]palmitate and is released into the medium. (**A**) CHP-100 cells were prelabelled with [^{14}C]palmitate (1 μCi/ml) for 24 h. After washing with RPMI-1640 containing 0.1% bovine serum albumin, cells were reincubated for the indicated times in label-free medium containing 30 μM C_6-Cer or vehicle. Lipids were then extracted, subjected to mild alkaline hydrolysis and resolved by HPTLC. Sphingolipids were visualized by iodine, scraped off into counting vials and their radioactivity determined. Lipid Z and lipid XY indicate the neosynthesized products shown in Figure 1B – C; Lc-Cer and SM indicate long-chain-ceramide and sphingomyelin, respectively. Data are means $\pm$ S.D. of four different determinations. Statistical significance: $^*P<0.01$, as compared to samples incubated in the presence of carrier only, as from Student's *t*-test. (**B**) Lipids were extracted from the culture media of CHP-100 cells maintained for 18 h either in the absence (lane 1) or presence of 30 μM C_6-Cer for (lane 2). Following mild alkaline hydrolysis lipids were resolved by solvent system II (see text) and detected according to Macala *et al* (1983). The arrows at the right indicate the migration of lipid Z, lipids XY and sphingom yelin (SM)

Neosynthesized glycosphingolipids are short- and long-chain GluCer

The neosynthesized monohexosylsphingolipids were potently labelled by [^{14}C]galactose (Figure 3), but barely by [^{14}C]glucose (not shown); although this result could suggest that neosynthesized lipids were galactosylceramides, we observed that D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), reported to inhibit GluCer but not galactosylceramide synthesis (Hakomori, 1996), was able to fully prevent C_6-Cer-induced monohexosylceramide formation at a 30 μM concentration (Figure 3). Since it is well known that, after isomerization, [^{14}C]galactose can efficiently label GluCer (Lavie *et al*, 1997), we further investigated the identity of the sugar mojety in neosynthesized lipids by bi-dimensional HPTLC analysis on borate-impregnated silica-gel plates (Kean, 1966). As shown in Figure 4, lipids XY comigrated with a naturally occurring GluCer standard in both dimensions; on the other hand, all the newly formed products migrated faster than galactosylceramide standards in the first dimension. These results clearly indicate that accumulating lipids are short- and long-chain GluCer. Remarkably, although GluCer is the earliest intermediate along the pathway of higher-order glycosphingolipids synthesis, we could not detect enhancement of [^{14}C]galactose incorporation into higher-order total neutral glycosphingolipids or gangliosides in C_6-treated cells (not shown).

Inhibition of GluCer synthesis potentiates ceramide-induced apoptosis

We studied whether inhibition of GluCer accumulation, due to cell treatment with PDMP affected C_6-Cer-induced apoptosis.

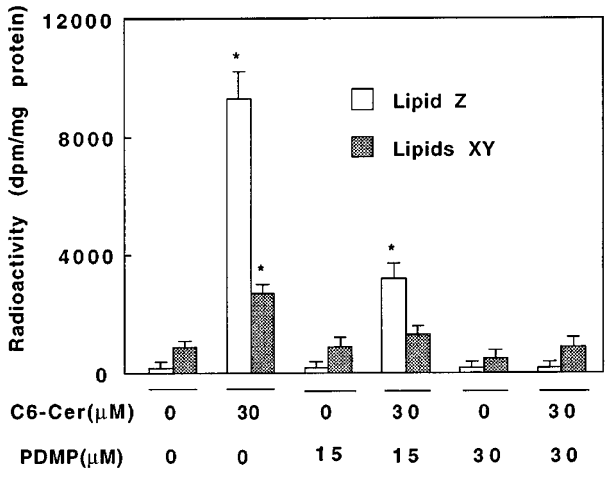

Figure 3 PDMP blocks [^{14}C]galactose incorporation into neosynthesized monohexosylsphingolipids. CPH-100 cells were incubated with growth medium containing 1 μCi/ml [^{14}C]galactose and 30 μM C_6-Cer or vehicle, either in the absence or presence of PDMP at the indicated concentrations. After 18 h, the neosynthesized products migrating as a doublet (Lipids XY) or as a single band (Lipid Z) (see Figure 1) were visualized by iodine, scraped off into counting vials and their radioactivity determined. Data are means $\pm$ S.D. of four different determinations. Statistical significance: $^*P<0.01$, as compared to samples labelled in the presence of carrier only, as from Student's *t*-test

As shown in Figure 5, PDMP, administered for 18 h up to the concentration of 30 μM, was not toxic to CHP-100 cells; this compound, however, dose-dependently potentiated the apoptotic effect induced by 30 μM C_6-Cer (Figure 5A). Moreover, when the inhibitor was administered at a 30 μM concentration it was able to allow apoptosis induction already at a 15 μM C_6-Cer concentration (Figure 5B). Figure 6 shows that 30 μM PDMP administered alone for 18 h to [^{14}C]palmitate-labelled cells elicited only an average 40% increase of Lc-Cer labelling over basal. Remarkably, 30 μM PDMP markedly potentiated accumulation of labelled Lc-Cer after exposure of [^{14}C]palmitate-labelled cells to C_6-Cer. In the absence of PDMP, 30 μM C_6-Cer elicited a fivefold increase of [^{14}C] Lc-Cer accumulation over basal; the increase was about eightfold in the presence of 30 μM PDMP.

Discussion

Herein we have investigated C_6-Cer metabolism in CHP-100 neuroepithelioma cells, in order to assess whether any derivative of the above mentioned lipid could be involved in apoptosis modulation. A first observation, however, was that, in the system presently studied, C_6-Cer uptake associated with elevation of Lc-Cer levels. Endogenous ceramide generation has been previously observed after C_2-Cer and C_6-Cer administration to rat fibroblasts (Gòmez-Muñoz *et al*, 1995); in addition, it has been reported that C_8-Cer elevates endogenous ceramide levels in Madin-Darby canine kidney cells, by inhibiting phosphocholinetransferase involved in sphingomyelin synthesis (Abe *et al*, 1992). While the routes through which Lc-Cer is produced in CHP-100 cells remain to

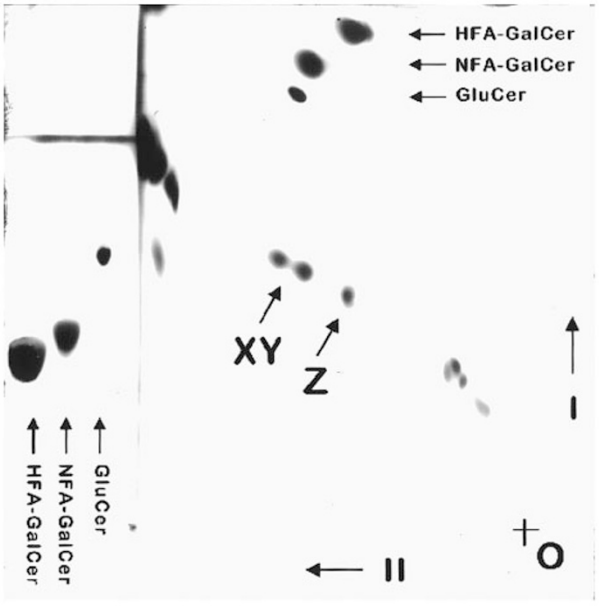

Figure 4 Identification of neosynthesized monohexosylsphingolipids as glucosyl ceramides by two-dimensional HPTLC analysis on borate-impregnated plates. CHP-100 cells were incubated with 30 μM C_6-Cer for 18 h. Lipids were then extracted, subjected to mild alkaline hydrolysis and resolved by two-dimensional HPTLC on borate-impregnated silica-gel G plates. Lipid detection was carried out according to Macala *et al* (1983). The arrows at the upper and left borders indicate the migration of standards of glucosylceramide (GluCer), non-hydroxy fatty acid galactosylceramide (NFA-GalCer) and hydroxy fatty acid galactosylceramide (HFA-GalCer) from bovine brain

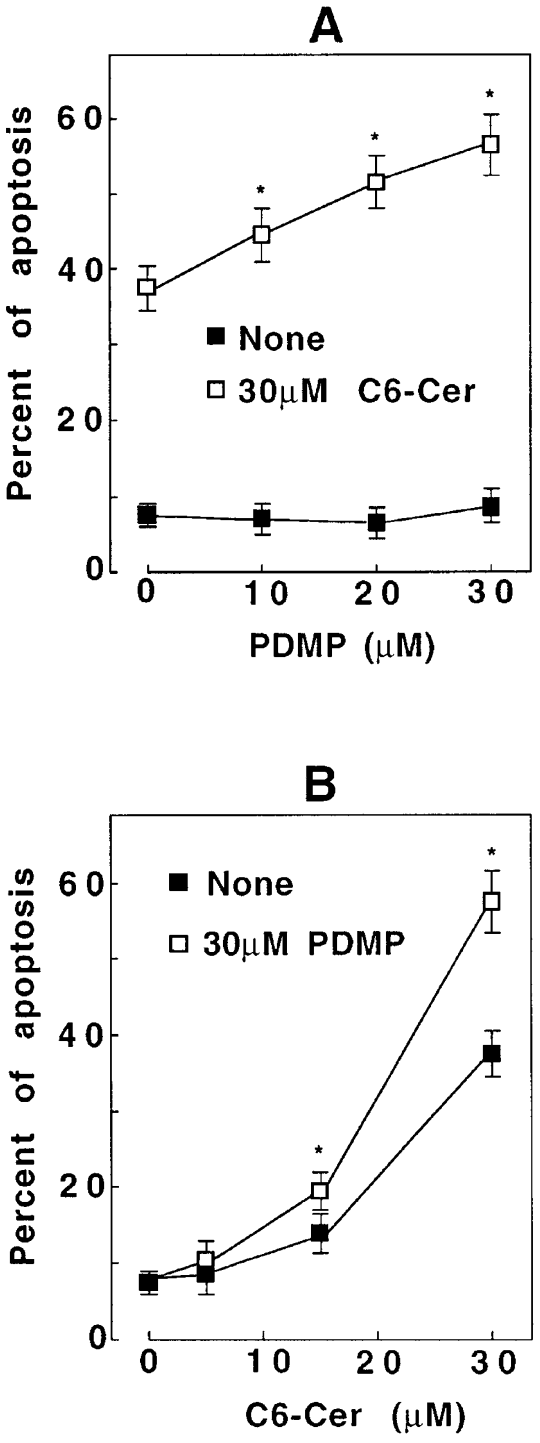

Figure 5 PDMP potentiates C_6-Cer-induced apoptosis. CHP-100 cells were incubated for 18 h with vehicle or 30 μM C_6-Cer, in the presence of PDMP at the indicated concentrations (**A**) or with vehicle or 30 μM PDMP and C_6-Cer at the indicated concentrations (**B**). Apoptosis was determined by flow cytometric analysis. Results are means $\pm$ S.D. of four different experiments. Statistical significance: $^*P < 0.01$, as compared to samples treated with carrier only, as from Student's *t*-test

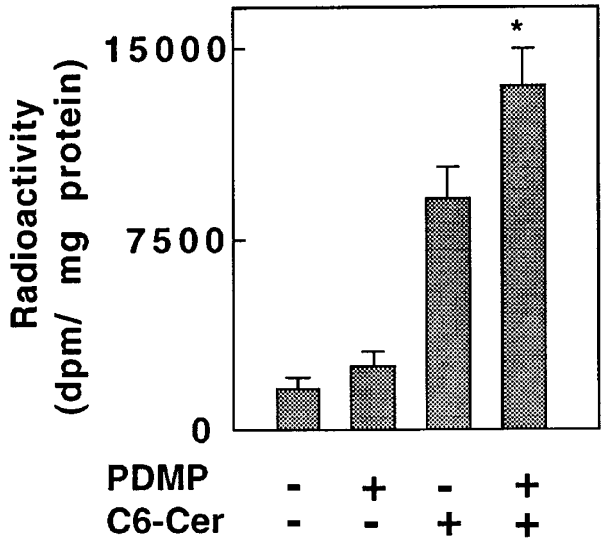

Figure 6 PDMP effects on endogenous ceramide levels in cells exposed or not to C_6-Cer. CHP-100 cells were prelabelled with [^{14}C]palmitate (1 μCi/ml) for 24 h. After washing with RPMI-1640 containing 0.1% bovine serum albumin, cells were reincubated for 18 h in label-free medium containing 30 μM C_6-Cer or vehicle ('+' and '$-$', respectively), in the presence or absence of 30 μM PDMP ('+' and '$-$', respectively). Lipids were then extracted, subjected to mild alkaline hydrolysis and resolved by HPTLC. Long-chain ceramide spots were visualized by iodine, scraped off into counting vials and their radioactivity determined. Data are means $\pm$ S.D. of four different determinations. Statistical significance: *$P < 0.01$, as compared to samples incubated in the presence of carrier only, as from Student's *t*-test

be fully elucidated, the magnitude of the phenomenon is such that it could relevantly contribute to apoptosis induction. We have shown that short- and long-chain ceramides are not appreciably metabolized to sphingosine, but are partially converted to GluCer; notably, block of this reaction, achieved by the GluCer synthase inhibitor PDMP, potentiated C_6-Cer-induced apoptosis, thus suggesting that GluCer synthesis provides a mean through which cells attempt to escape programmed cell death. Ceramide glucosylation could fulfil its anti-apoptotic function at least partly by downregulating intracellular ceramide levels; in line with this view we have shown that C_6-Cer-induced Lc-Cer elevation is potentiated by PDMP. Nonetheless, the possibility that GluCer itself might also function as an active lipid in counteracting apoptosis cannot be presently ruled out. In line with our results, recent studies have demonstrated that a subclone of MCF-7 breast cancer cells which is resistant to adriamycin displays higher levels of GluCer and synthesizes more rapidly this glycosphingolipid than the wild-type counterpart; moreover, inhibition of ceramide glucosylation restores cell sensitivity to adriamycin (Lavie *et al*, 1996; 1997).

It must be recalled that ceramide glucosylation provides the first step towards the synthesis of higher-order lactosylceramide-based neutral glycosphingolipids and gangliosides, some of which have been demonstrated to play an active role in apoptosis induction. Evidence for a relationship between globotriaosylceramide expression and apoptosis in B lymphocytes (Mangeney *et al*, 1995) and Burkitt lymphoma cells (Mangeney *et al*, 1993) has been provided. More recently, it has been shown that apoptosis induced in HuT78 human lymphoma cells by Fas ligation or short-chain ceramide administration is mediated by GD3 ganglioside synthesis and can be prevented by GluCer synthase inhibition (De Maria *et al*, 1997). Intriguingly, in our system, we could not detect substantial metabolism of newly synthesized GluCer to lactosylceramide or higher-order glycosphingolipids. The basis of this phenomenon are presently unknown. In the case of short-chain neutral glycosphingolipids we entertained the possibility that these products could be lost during lipid extraction, owing to preferential partion into the water/methanol phase; on the other hand, analysis of the Folch upper phase demonstrated that this was not the case (not shown). Intriguingly, short-chain Cer metabolism to GluCer but not to higher-order glycosphingolipids has also been reported in hyppocampal neurons (Harel and Futerman, 1993; Boldin and Futerman, 1997); herein, in addition, we could not even detect substantial accumulation of glycosphingolipids derived from GluCer bearing long-chain fatty acids. Thus, a possibility to be entertained is that, in the system presently studied, GluCer synthesis that follows the abrupt increase of ceramide levels is sequestered from the routes that lead to lactosylceramide and higher-order glycosphingolipid synthesis. Taken together, the present results and those reported by other groups suggest that ceramide glucosylation initiates a multifunctional pathway, such that programmed cell death can be triggered or prevented, depending on the cell system and/or on the final products of ceramide metabolism.

Materials and Methods

Materials

CHP-100 human neuroepithelioma cells were obtained through the courtesy of Prof. G Melino (University of L'Aquila, Italy). D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, bovine brain ceramide and galactocerebrosides as well as human glucocerebrosides were from Sigma Chemical Co. (St. Louis, MO, USA); higher-order neutral glycosphingolipids, sphingosine and C_6-Cer were from Calbiochem Novachem Co. (La Jolla, CA, USA). D-[U-^{14}C]galactose (295 mCi/mmol), [^{14}C]palmitic acid (55.3 mCi/mmol) and the chemoluminiscence ECL detection system were from Amersham Corp. (Bucks., UK). HPTLC silica gel 60 plates were from Merck, Darmstadt, Germany.

Cell culture and apoptosis evaluation

CHP-100 cells were grown at 37°C in RPMI-1640 medium, supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% (v/v) CO_2. C_6-Cer and other chemicals were administered to cells in complete growth medium. Apoptosis was monitored by evaluation of the pre-G_1 cell population, after flow cytometric analysis. Cells were detached from the plates by trypsin treatment and centrifuged at 300 $\times$ *g* for 5 min; pellets were washed with phosphate-buffered saline (pH 7.4), placed on ice and overlaid with 0.5 ml of a solution containing 50 μg/ml propidium iodide, 0.1% Triton X-100 and 0.1% sodium citrate. After gentle resuspension

in this solution cells were left at 4°C for 30 min at least, in the absence of light, before analysis. Propidium iodide-stained cells were analyzed using a FACScan Flow Cytometer (Becton-Dickinson, CA, USA); fluorescence was measured between 565 and 605 nm. The data were acquired and analyzed by the Lysis II program (Becton-Dickinson, CA, USA).

Lipid extraction and separation

Lipids were extracted according to the methods of Bligh and Dyer (1959) or Folch *et al* (1957); the latter method was preferred for extraction of high order neutral glycosphingolipids and gangliosides. Lipid extracts were subjected to mild alkaline hydrolysis with 0.1 M methanolic KOH for 1 h at 37°C and, after re-extraction, the chloroformic phase was analyzed by HPTLC. Ceramide and sphingosine separation was achieved by developing samples in n-hexane/diethylether/acetic acid (25 : 75 : 1, vol/vol) for full plate height, followed by development in same direction in chloroform/methanol/0.7 N NH_4OH (20 : 5 : 0.5, vol/vol), up to 3 cm from the top, and again in the same direction in n-hexane/diethylether/acetic acid (25 : 75 : 1, vol/vol) for full plate height (solvent system I). Monodimensional resolution of neutral glycosphingolipids was achieved by developing samples in chloroform/methanol/water (65 : 25 : 4, vol/vol) (solvent system II). Two-dimensional lipid analysis was carried out on HPTLC plates dipped in 2.5% boric acid in methanol and activated by heating at 110°C for 30 min. Samples were developed in the first dimension in chloroform/methanol/25% NH_4OH/water (65 : 35 : 4 : 4, vol/vol) and in the second dimension in chloroform/acetone/methanol/acetic acid/water (50 : 20 : 10 : 10 : 5, vol/vol). Two-dimensional separation was also employed for sphingomyelin resolution. Unless otherwise mentioned, lipids were visualized by dipping the plates in a solution of 3% cupric acetate in 8% phosphoric acid and heating in an oven for 15 min at 180°C, according to Macala *et al* (1983). For glycosphingolipid detection, plates were sprayed with a solution made of 10.5 ml methanolic α-naphthol (5%, w/v), 6.5 ml concentrated sulphuric acid, 40.5 ml ethanol and 4 ml water, followed by hearing for 10 min at 90°C, as reported (Ando and Saito, 1987). Fatty acids were transmethylated and analyzed by a Perkin Elmer 8310 gas chromatograph, equipped with a Supelcowax 10 fused silica capillary column, as previously described (Spinedi *et al*, 1987). Total gangliosides were isolated according to Williams and McCluer (1980).

[^{14}C]palmitate and [^{14}C]galactose incorporation into sphingolipids

Cells grown to subconfluence in 35 mm plates were labelled with [^{14}C]palmitic acid (1 μCi/ml) for 24 h. After washing with RPMI plus 0.1% bovine serum albumin, 30 μM C_6-Cer was added for the indicated times. [^{14}C]Galactose (1 μCi/ml) was administered to cells concomitantly with C_6-Cer and/or PDMP at the concentrations reported in the text. After extraction and separation, lipids were visualized under iodine and spots scraped off from the plates into counting vials for radioactivity determination. Protein determination was carried out according to Lowry *et al* (1951). Lipid phosphate was measured according to Marinetti (1962).

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References

Abe A,A Wu D, Shayman JA and Radin NS (1992) Metabolic effects of short-chain ceramide and glucosylceramides on sphingolipids and protein kinase C. Eur. J. Biochem. 210: 765 – 773

Ando S and Saito M (1987) TCL and HPTLC of phospholipids and glycolipids in health and disease. In: Kuksis A, (ed). Chromatography of lipids in biomedical research and clinical diagnosis. (Elsevier Science Publisher BV: Amsterdam) pp. 266 – 305

Bligh EC and Dyer WJ (1959) A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911 – 917

Boldin S and Futerman AH (1997) Glucosylceramide synthesis is required for basic fibroblast growth factor and laminin to stimulate axonal growth. J. Neurochem. 68: 882 – 885

Casaccia-Bonnefil P, Carter BD, Dobrowsky RT and Chao MV (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 383: 716 – 719

Cifone MG, DeMaria R, Roncaioli P, Rippo MR, Azuma M, Lanier LL, Santoni A and Testi R (1994) Apoptotic signaling through CD95 (Fas/Apo-1) activates in acidic sphingomyelinase. J. Exp. Med. 177: 1547 – 1552

De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR and Testi R (1997) Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. Science 277: 1652 – 1655

Dobrowsky RT, Werner MH, Castellino AM, Chao MV and Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265: 1596 – 1599

Folch J, Lees M and Sloane-Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 266: 497 – 509

Gòmez-Muñoz A, Waggoner DW, O'Brien L and Brindley DN (1995) Interaction of ceramides, sphingosine, and sphingosine-1-phosphate in regulating DNA synthesis and phospholipase D activity. J. Biol. Chem. 270: 26318 – 26325

Hakomori S. (1996) Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. Cancer Res. 56: 5309 – 5318

Hannun YA (1996) Functions of ceramide in coordinating cellular responses to stress. Science 274: 1855 – 1859

Harel R and Futerman AH (1993) Inhibition of sphingolipid synthesis affects axonal outgrowth in cultured hippocampal cells. J. Biol. Chem. 268: 14476 – 14481

Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA and Grant S (1994) Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc. Natl. Acad. Sci. USA 91: 73 – 77

Kean EL (1966) Separation of gluco- and galactocerebrosides by means of borate thin-layer chromatography. J. Lipid Res. 7: 449 – 452

Kim M, Linardic CM, Obeid LM and Hannum YA (1991) Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor and γ-interferon. J. Biol. Chem. 266: 484 – 489

Lavie Y, Cao H, Bursten SL, Giuliano AE and Cabot MC (1996) Accumulation of glucosylceramides in multidrug-resistant cancer cells. J. Biol. Chem. 271: 19530 – 19536

Lavie Y, Cao H, Volner A, Lucci A, HanT-Y, Geffen V, Giuliano AE and Cabot MC (1997) Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. J. Biol. Chem. 272: 1682 – 1687

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265 – 275

Macala LJ, Yu RK and Ando S (1983) Analysis of brain lipids by high performance thin-layer chromatography and densitometry. J. Lipid Res. 24: 1243 – 1250

Mangeney M, Lingwood CA, Taga S, Caillou B, Tursz T and Wiels J (1993) Apoptosis induced in Burkitt's lymphoma cells via Gb3/CD77, a glycolipid antigen. Cancer Res. 53: 5314 – 5319

Mangeney M, Rousselet G, Taga S, Tursz T and Wiels J (1995) The fate of human CD77+ germinal center B lymphocytes after rescue from apoptosis. Mol. Immunol. 32: 333 – 339

Martinetti GV (1962) Chromatographic separation, identification and analysis of phosphatides. J. Lipid Res. 3: 1 – 20

Merril AH and Jones DD (1990) An update of the enzymology and regulation of sphingomyelin metabolism. Biochim. Biophys. Acta 1044: 1 – 12

Obeid LM, Linardic CM, Karolak LA and Hannun YA (1993) Programmed cell death induced by ceramide. Science 259: 1769 – 1771

Ohta H, Sweeney EA, Masamune A, Yatomi Y, Hakomori S and Igarashi Y (1995) Induction of apoptosis by sphingosine in human leukemic HL-60 cells: a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation. Cancer Res. 55: 691 – 697

Peña LA, Fuks Z and Kolesnick R (1997) Stress-induced apoptosis and the sphingomyelin pathway. Biochem. Pharmacol. 53: 615 – 621

Rabizadeh S, Oh J, Zhong L, Yang J, Bitler CM, Butcher LL and Bredesen DE (1993) Induction of apoptosis by the low-affinity NGF receptor. Science 261: 345 – 348

Spinedi A, Rufini S and Luly P (1987) Lipid composition and temperature adaptation of the nervous system of the leech Hirudo medicinalis L. J. Neurochem. 49: 45 – 49

Spinedi A, Amendola A, Di Bartolomeo S and Piacentini M (1998) Ceramide-induced apoptosis is mediated by caspase activation independently from retinoblastoma protein post-translational modification. Biochem. Biophys. Res. Commun. 243: 852 – 857

Sweeney EA, Sakakura C, Shirahama T, Masamune A, Ohta H, Hakomori S and Igarashi Y (1996) Spingosine and its methylated derivative N,N-dimethyl-sphingosine (DMS) induce apoptosis in a variety of human cancer cell lines. J. Cancer 66: 358 – 366

Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, Debatin KM and Krammer PH (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245: 301 – 305

Williams MA and McCluer RH (1980) The use of Sep-Pak C_{18} cartridges during the isolation of gangliosides. J. Neurochem. 35: 266 – 269