Natural C18:0-ceramide Induces Cellular Sphingolipid Accumulation And Apoptosis

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Abstract

Exogenous ceramides induces cell differentiation, cell cycle arrest and apoptosis in various cell types. However, the permeable short-chain ceramides have drawn more attention. In this study the effect of natural N-stearoyl-D-erythro-sphingosine (C18-ceramide) as well as short chain N-acetylsphingosine (C2-ceramide) and bovine brain non-hydroxy fatty acid ceramides on human keratinocyte growth and apoptosis and cellular sphingolipid accumulation was compared. Cells were cultured in the presence of 5-60 μM of ceramides or ethanol/dodecane for the 24 h. No significant release of lactate dehydrogenase was observed when the HaCaT cells were incubated with up to 60 μM of natural and C2-ceramides. Culturing of the cells in the presence of C18-ceramide leads to the concentration-dependent increase of the content of ceramide and sphingomyelin in the cells, reduction of keratinocyte proliferation, induction of cell apoptosis, and does not change the glucosylceramide content in the cells. It has been determined that the glucosylceramide synthase inhibitor D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-pr opanol alone as well as combined with C18-ceramide was not toxic for HaCaT cells and did not enhanced the C18-ceramide-induced keratinocyte apoptosis. Comparison of biological activity of the ceramides species showed that natural C18-ceramide is the most potent inhibitor of the HaCaT cell growth and an inducer of cellular sphingolipid accumulation and cell apoptotic death.

Introduction

Ceramides are an important regulator of various stress responses and growth mechanisms. The formation of ceramides from the hydrolysis of sphingomyelin (SM) or from de novo pathways is observed in response to inducers of stress such as tumour necrosis factor-α, γ-interferon, interleukin-1, ultraviolet light, heat, chemotherapeutic agents, etc [1-3]. The addition of exogenous short-chain ceramides or the enhancement of cellular levels of ceramides induces cell differentiation, cell cycle arrest, apoptosis, or cell senescence in various cell types.

Biological effects were observed with various ceramides and ceramide metabolites. However, the permeable short-chain ceramides have drawn more attention. It was demonstrated that two new cell-permeable ceramide analogues, N-thioacetylsphingosine and 4-dodecanoylamino-decan-5-ol, as well as well-investigated ceramide analogue N-acetylsphingosine (C2-ceramide) inhibited proliferation of HaCaT cells and induced the programmed cell death in the human epidermis [4,5]. It was demonstrated that C2-ceramide induces apoptosis of the transformed human keratinocytes, whereas C2-dihydroceramide does not have such an effect [6]. The fact that ceramides induce apoptosis of keratinocytes raises the possibility that intracellular ceramide which is increased with differentiation of the epidermis might be involved in terminal differentiation, a specialized form of apoptosis of keratinocytes. It has been demonstrated that N-hexanoyl sphingosine (C6-ceramide) induces apoptosis of HaCaT keratinocytes efficiently through its hydrolysis to sphingosine and its reacylation to long-chain sphingolipids, such as N-stearoyl-D-erythro-sphingosine (C18-ceramide), on apoptosis and HaCaT cell proliferation have not been examined. However, using the human MDA435/LCC6 and mouse macrophage J774 cells it was demonstrated that acyl chain length affected the ceramide pro-apoptotic activity [8]. It has been found that for C6-, C8-, C10-, C14- and C24-ceramide, the chain length was inversely proportional to the cytotoxic activity. C6-ceramide-induced apoptosis was associated with cell cycle arrest in the G2/M phase, decreased extracellular signal-regulated kinase (ERK1/2) signalling and activation of the cell cycle regulatory protein retinoblastoma (pRb) [9]. Treatment of PC-3 cells with natural SM and C18-ceramide did not alter cell cycle distribution, pRb or ERK1/2 activation. Taken together, these results suggest that natural and synthetic sphingolipids induce apoptosis in PC-3 cells via distinct signalling mechanisms and potencies.

The long-chain (C18, as well as C12, C16 and C24)
but not short-chain ceramides suppress mitochondrial permeability transition pore activity at physiological concentrations [10]. These studies raise the possibility that long-chain ceramides promote apoptosis at the level of the mitochondria by causing permeability transition pore closure in the liver cells. Only one specific ceramide, C18-ceramide, was selectively down-regulated in the majority of tumour tissues [11]. It has been determined that increased generation of C18-ceramide by the differentiation factor 1 (mUOG1), a mouse homologue of longevity assurance gene 1 (mLAG1), inhibited cell growth and induced the apoptotic cell death by mitochondrial dysfunction. In this work we compare the possible physiological role of certain ceramides, such as C2-ceramide and brain non-hydroxy fatty acid ceramides as well as natural skin C18-ceramide in epidermal growth and apoptosis. For this purpose, the human keratinocyte cell line HaCaT was treated with different concentrations of the ceramides and their effect on cellular sphingolipid accumulation, cells proliferation and death was investigated.

Methods

Materials used: N-Acetyl-D-erythro-sphingosine, (C2-ceramide), purity 98 %, and brain non-hydroxy fatty acid ceramides as well as natural skin C18-ceramide were obtained from Department of Chemical Technology, Kaunas University of Technology (Lithuania). Cell death detection ELISAPlus kit from Boehringer Mannheim (Germany). Cytotoxicity Detection kit (LDH) from Roche (Germany). Media and culture reagents were obtained from Seromed-Biochrom (Berlin, Germany). Plastic culture dishes were from Nunc (Wiesbaden, Germany).

The spontaneously immortalized human keratinocyte cell line HaCaT [12] was grown in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 0.35 g/L glutamine, 100,000 IU/L penicillin and 0.1 g/L streptomycin in plastic culture dishes. Ceramides were added to the culture medium from a 10 mg/mL ethanol solution (C2-ceramide) or 10 mg/mL ethanol/dodecane 49:1 (v/v) (foreskin and brain ceramides) and cells were cultured during 24 h. Ethanol or ethanol/dodecane were added to controls in the respective concentration and did not significantly influence viability, proliferation and apoptosis of HaCaT cells. Cytotoxicity of the ceramides was determined by the release of lactate dehydrogenase (LDH) from the cytosol of damaged cells into the culture medium as described previously [13]. Cell proliferation was determined by staining cells with crystal violet [13]. Cell apoptosis was determined quantitatively by using the Cell death detection ELISAPLus assay according to the manufacturer’s instructions.

For lipid extraction from keratinocyte cells were homogenized with 0.9% solution of sodium chloride. Lipids were extracted for 24 h at 37oC in each of three solvent mixtures: chloroform/ethanol/water 1:2:0.5 (v/v/v), chloroform/ethanol 1:1 (v/v) and chloroform/ethanol 2:1 (v/v). The chloroform phase was collected and dried under N2 at 37oC. Acylglycerolipids were hydrolyzed during 1h of incubation at 37oC in chloroform/methanol 1:1 (v/v) containing 0.1M KOH. The remaining lipids were re-extracted and used for the HPTLC separation. Lipid extracts was applied to high performance thin–layer Silica Gel 60 plates (Merck Darmstadt, Germany). Plates were washed with chloroform/methanol (9:1, v/v) before sample application. For the separation of ceramides and SM, the plates with loaded lipid extract were developed with diethyl ether. When the solvent front reached the top of the plate, the plate was removed from the chamber, dried, and developed again with chloroform/methanol/water (40:10:1, v/v). In this case, the solvent front was allowed to reach 2/3 of the plate length. The developed chromatograms were sprayed with 10% (w/v) cupric sulfate hydrate in 8% (w/v) phosphoric acid and charred on a heat block at 180?C for 60 min. After cooling the chromatograms were scanned with photodensitometer (Model GS-700 Imaging Densitometer) for quantization.

One-way analysis of variance (ANOVA) procedures was used to assess significant differences among treatment groups. Student’s t-test was used for paired observations.

Results

Exposure of HaCaT cells to natural foreskin C18-ceramide or the mixture of brain ceramides leads to the concentration-dependent increase of ceramide and SM contents in the cells (Fig. 1A, 2B). Cell culturing in the presence of the C2-ceramides did not change the level of endogenous sphingolipids in the keratinocytes. To study the effect of natural and synthetic ceramides on keratinocytes proliferation the HaCaT cells were treated with either C18-ceramide or C2-ceramide. It has been determined that the ceramides induce the inhibition of cell proliferation in a
concentration-dependent manner (Fig. 3). The most potent inhibitor of the HaCaT cell growth was C18-ceramide. The anti-proliferative effects of natural C18-ceramide were not due to nonspecific, detergent-like cytotoxicity. No significant release of LDH was observed when the cells were incubated up to 60 µM of C18-ceramide, brain ceramides or C2-ceramide (Fig. 4) indicating that membrane integrity was not impaired.

In another set of experiments, we tested the ability of ceramides to induce apoptosis. As shown in Fig. 5, foreskin C18-ceramide or C2-ceramide induced apoptosis after 24 h in a concentration-dependent manner. A significant increase of apoptotic cells was seen at ceramide concentration levels of 15-60 µM. C18-ceramides induced an apoptotic cellular response, which was greater in magnitude than that observed with C2-ceramide at all used concentrations. A maximum apoptotic response of approximately 800% and 250% was observed after 24 h treatment with 60 µM of C18- and C2-ceramides, respectively.

Discussion

Some studies have demonstrated that alterations in the cellular ceramide level from either exogenous short-chain ceramide (C2- and C8-ceramide), or exogenous bacterial sphingomyelinase inhibit DNA synthesis [15], and induce cultured human keratinocytes apoptosis [15, 16]. It has been determined that DNA-damaging agents, mitomycin C and daunorubicin, and apoptosis-inducing ligands, tumor necrosis factor alpha (TNF-alpha) and the TNF-related apoptosis-inducing ligand (TRAIL), affect the C18-ceramide level in the mammalian cells [17]. C18-ceramide, was selectively down-regulated in the majority of tumour tissues [11], while increased generation of C18-ceramide by the differentiation factor 1 (mUOG1) inhibited cell growth. The results presented in this study demonstrate that exogenous natural (skin and brain) ceramides increase levels of cell associated ceramides and SM. The effect of exogenous lipids on sphingolipid accumulation is concentration- and ceramide structure-dependent. It has been determined that natural ceramides lead to a more pronounced effect on sphingolipid accumulation as compared with C2-ceramide. Taking into account, that ceramides are the precursor of SM synthesis via SM synthase in the mammalian cells it could be concluded that the increase of a long-chain ceramide level in the cell culture media leads to the increase of a ceramide level in the cells and their use in the SM synthesis. It has been shown previously that the addition of short chain C2- and C6-ceramides to cells mimics many of the biological responses of agonists in mammalian and yeast cells, and this approach has been employed to suggest possible roles for endogenous ceramides in these processes [18, 19]. In addition, there are results suggesting a more direct relationship between exogenous and endogenous ceramides. It has been shown that treatment of Madin-Darby canine kidney cells with N-octanoyl sphingosine liposomes causes about 10-fold increase in the total ceramide levels [20]. Treatment of A549 human lung adenocarcinoma cells with cell-permeable C6-ceramide triggered a sustained endogenous ceramide generation in 24 h, which was inhibited by fumonisin B1 [21]. Furthermore, it has been shown recently that treatment of human skin fibroblasts and peripheral blood mononuclear cells with C6-ceramide induces acid sphingomyelinase expression at the mRNA level that correlates with apoptotic cell death [22]. Thus, there appear to be multiple mechanisms by which exogenous ceramides influence the cellular ceramide metabolism. These observations also raise the important question of whether downstream biological effects are mediated by short chain ceramides per se or if they are dependent on the generation of endogenous ceramides. In this study, we determined that exogenous short chain C2-ceramide did not induce the accumulation of endogenous long chain ceramides and did not change SM level in the HaCaT cell line. However, it remains to be determined whether ceramide long-chain species are involved in the anti-proliferative and apoptotic response of keratinocytes.

The results obtained demonstrated that ceramides studied induced the inhibition of cell proliferation in a concentration-dependent manner and that skin derived C18-ceramide was the most potent inhibitor of the HaCaT cell growth as compared with C2-ceramide. The anti-proliferative effects of C18-ceramide were not due to nonspecific, detergent-like cytotoxicity, since LDH release was not altered. Moreover, it has been determined that C18-ceramide as well as C2-ceramide induces apoptosis after 24 h in a concentration-dependent manner. However, C18-ceramide is a more potent inducer of HaCaT cell apoptotic death as compared with C2-ceramide possibly due to the more pronounced accumulation of natural ceramides in the cells. It has been determined that C18-ceramide as well as other long-chain natural ceramides in a dodecane/ethanol delivery system successfully utilized for ceramide delivery to sub-cellular organelles in situ [23, 24]. Besides, it was demonstrated that natural and synthetic ceramides induce apoptosis via distinct signalling mechanisms.
and potency [9]. C6-ceramide-induced apoptosis was associated with cell cycle arrest in the G(2)/M phase, decreased ERK1/2 signalling and activation of the cell cycle regulatory protein, pRb. Treatment of the PC-3 cells with C18-ceramide significantly induced apoptosis and did not alter cell cycle distribution, pRb or ERK1/2 activation. These results, therefore, suggest that the roles of endogenous long chain ceramides and exogenous short chain ceramides in apoptotic cell death might be distinct and/or cell line-specific. However, distinct roles of exogenous short chain and endogenous long chain ceramides in apoptotic cell death need to be further examined.

Conclusion(s)

Consequently, this study provides important data showing, for the first time, the cellular long chain ceramides accumulation and endogenous SM generation in response to exogenous C18-ceramide in the HaCaT cells. Also, the results presented in this study demonstrate an important role for endogenous C18-ceramide in the regulation of growth and apoptosis in the HaCaT cells. Taking into account, that natural long-chain ceramides are not as expensive as synthetic ones and do not have any risk for human being as brain products [25] reasonable assumption can be made that skin ceramides is a new tool for a novel ceramide therapy.

Authors Contribution(s)

NB and CG conceived of the study and participated in its design, coordination, and manuscript preparation. LH participated in data collection and performed the statistical analysis. MB and JL isolated and analysed skin C18-ceramide.

References

Illustrations

Illustration 1

Effect of natural and synthetic ceramides on ceramide (A) contents in the HaCaT cells. *P < 0.05 vs. control.

![Illustration 1](image1.png)

Illustration 2

Effect of natural and synthetic ceramides on sphingomyelin (B) contents in the HaCaT cells. *P < 0.05 vs. control.

![Illustration 2](image2.png)
Illustration 3

Effect of natural and synthetic ceramides on HaCaT cell proliferation. *P < 0.05 vs. control.

Illustration 4

Effect of natural and synthetic ceramides on the lactate dehydrogenase release from the HaCaT cells.
Illustration 5

Effect of natural and synthetic ceramides on the HaCaT cell apoptosis. *P < 0.05 vs. control.
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