Original Investigation

Exogenous Ceramide Treatment Induce Death and Cytotoxicity in Glioma Cells

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ABSTRACT

AIM: To evaluate the cytotoxic and proapoptotic effects of C6 ceramide on the C6 rat glioma cell line.

MATERIAL and METHODS: The C6 rat glioma cell line was evaluated. Using a confocal microscope and the appropriate software, the cytotoxic effects of C6 ceramide were identified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) colorimetric experiments. Transmission electron microscopy (TEM) was utilized to examine the ultrastructural changes following treatment with IC_{50} concentrations of C6 ceramide

RESULTS: Condensation and fragmentation of nuclei and DNA laddering was observed, indicating apoptotic cell death. C6 ceramide induced apoptosis and effectively caused cytotoxicity in the C6 glioblastoma cells. MTT assay demonstrated >90% cell death after short-term application of C6 ceramide, confirming its apoptosis-triggering effect. Apoptosis was also confirmed via confocal microscopy and TEM.

CONCLUSION: Glioblastoma cells undergo apoptosis when exposed to C6 ceramide, which makes it a potential chemotherapeutic agent for the treatment of this aggressive brain cancer.

KEYWORDS: Acid ceramidase, Apoptosis, Ceramide, Glioma, Glioblastoma multiforme

ABBREVATIONS: aCDase: Acid ceramidase, **ATCC:** American Type Culture Collection, **BBB:** Blood brain barrier, **DMEM:** Dulbecco's modified Eagle's medium, **DMSO:** Dimethyl sulfoxide, **FBS:** Fetal bovine serum, **GBM:** Glioblastoma multiforme, **MTT:** 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide, **PBS:** Phosphate buffered saline, **SIP:** Sphingosine-1-phosphates, **TEM:** Transmission electron microscopy

■ INTRODUCTION

In 2021, the World Health Organization classified brain tumors into several categories based on their histological and molecular characteristics. The most malignant central nervous system tumor, glioblastoma multiforme (GBM), was included in the astrocytic tumors category (10). GBM is highly invasive and its removal is challenging for surgeons and patients. Despite the current therapy approaches, namely surgery, radiotherapy, and chemotherapy, GBM continues to have a poor prognosis and requires novel effective agents and treatment approaches with lesser side effects. Currently, the standard treatment protocols for GBM are surgical resection and radiotherapy; however, they do not significantly change the mean survival of the patients. Recurrence or progression of GBM occurs due to the development of resistance to treatment agents in cancer stem cells. Thus, the disease remains incurable and requires new strategies and therapeutics for its treatment (1,4).

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Cell lipid metabolism produces numerous biological effects in a range of cellular pathways. Members of this class, such as sphingolipids, are structural molecules in cellular membranes that control several cellular processes such as cell division, cell growth and divergence, and programmed cell death. Sphingolipids are made up of a sphingosine backbone, fatty acids, and various polar head groups. Some sphingolipid members, such as sphingomyelins, sphingosine-1-phosphates (SIPs), and ceramides, play critical roles in the function of cellular membranes (8). Ceramides of cellular lipids are bioactive molecules that can alter cellular processes by altering membrane properties via the inhibition of receptors, protein kinases, and ion transporters as well as signal transduction. Ceramides are the main mediators of the antiproliferative cellular responses such as apoptosis and growth inhibition (14). This indicates that ceramides have a dual action in sphingolipid metabolism by being involved in the synthesis and catabolism of sphingolipids (2,19). Ceramides have amide-linked acyl chains and sphingosine bases. They may be produced via several mechanisms, including ceramide synthesis, de novo synthesis, and sphingomyelin hydrolysis. In the de novo synthesis pathway, serine palmitoyl transferase catalyzes the production of ceramides from serine and palmitoyl CoA. (2,7,11). In contrast to ceramides, SIPs induce cell proliferation, and the balance between the two plays a crucial role in determining whether a cell will undergo apoptosis (17).

Intracellular ceramide levels in tumor cells increase during apoptosis. Thus, the increase and/or accumulation of intracellular ceramide levels might be a good strategy for the treatment of tumors (9,11,16). Apoptosis, a physiological programmed cell death, plays a critical role in maintaining the normal development and homeostasis of normal cells. Thus, abnormal apoptotic processes can cause disorders such as cancers (13). Therefore, accumulation of ceramides in cells under stressful conditions and programmed cell death are reportedly potent therapeutic targets for cancer treatment(3). The pro-apoptotic effect of ceramides was first reported in leukemia cells, which lead to the current understanding that the principal apoptosis-initiating factor in several tumor cells is the ceramide-signaling pathway (11,13).

The short chain ceramide C6 is a cell-permeable lipid mediator and second messenger in neurons. Moreover, C6 ceramide has a unique characteristic of being able to cross the blood–brain barrier (BBB). Thus, it is considered to have a great potential for cancer therapy. In numerous cancer cell lines, C6's anticancer action has been demonstrated in conjunction with another short chain ceramide (C2) (3). Herein, we aimed to analyze the effects of C6 ceramide on the C6 rat glioma cell line and assess its cytotoxic and proapoptotic effects as well as indirectly understand the signaling pathways that are engaged during apoptosis.

■ MATERIAL and METHODS

Materials

A C6 rat glioma cell line was acquired from the American Type Culture Collection (ATCC; Rockville, MD, USA). Fetal bovine serum (FBS), penicillin/streptomycin, dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), and these substances were obtained from Sigma (St. Louis, MO, USA). The C6 ceramide was obtained from Cayman Chemicals (Ann Arbor, MI, USA).

Cell Culture

The C6 cells were added to the DMEM (Sigma, St. Louis, MO, USA) along with 10% FBS and 1% penicillin/streptomycin (100 U/mL-100 g/mL) (Sigma, St. Louis, MO, USA). This was cultured at 37°C in a humid environment with 95% O_2 and 5% CO_2. The cells were routinely passaged every third. Confluent cells were used in each experiment.

Cytotoxicity Assay

The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay was performed to determine the cytotoxic effects of C6 ceramide on the C6 rat glioma cell line. A stock solution of C6 ceramide (Cayman Chemical, Ann Arbor, MI, USA) was obtained by dissolving it in DMSO (Sigma, St. Louis, MO, USA), and further dilutions were obtained by adding the DMEM. In flat-bottomed 96-well plates with different C6 ceramide concentrations (3.125, 6.25, 12.5, 25, 50, and 100 mM with a final DMSO concentration of 0.01 %/well), C6 cells ($5x10^3$ cells/well) were cultured and incubated for 24 h and 48 h at 37°C in triplicates in a humid environment with 5% CO_2. The control cells were untreated C6 cells grown without C6 ceramide. Subsequently, 20 mL of MTT solution (5 mg/mL diluted in phosphate buffered saline [PBS]) was added to each well, and cells were incubated for another 4 h. After the formazan crystals had completely dissolved, the media were changed with 100 mL of DMSO per well, and the absorbances were measured using a microplate reader at 590 nm (BioTek Instruments HTX-Synergy, USA). The absorbances of the control C6 cells were used to calculate the viability percentages, which allowed for the detection of the IC50 values. The IC50 values were employed in all subsequent experiments.

Confocal Microscopy Analysis for Morphological Changes

C6 cells were mounted on coverslips in six-well plates and incubated at 37°C for 24 hours to determine the IC50 values of C6 ceramide. The C6 cells-containing coverslips were washed in PBS (Invitrogen) after the liquid portion was removed. Thereafter, the cells were double-stained with Alexa Fluor-488 phalloidin and acridine orange after being fixed in 2% glutaraldehyde for 15 min at room temperature. A confocal microscope (Leica TCS-SP5 II;) and the necessary software were used to analyze the cell morphology following staining. Fragmented DNA, condensed nuclei, cell membrane changes, and fragmented cytoskeleton were the morphological changes of interest to elucidate the apoptotic effects of C6 ceramide on C6 rat glioma cells.

Transmission Electron Microscopy Analysis

Transmission electron microscopy (TEM) was used to study the ultrastructural alterations in 25 cm² cell culture flasks caused by treatment with IC50 doses of C6 ceramide at 37°C for 24 h. The untreated C6 cells served as the control group. Every cell sample was fixed individually in 2% glutaraldehyde,

followed by 2% osmium tetroxide. Following cell dehydration in graded ethanol and embedding in Epon 812 epoxy, the cells were treated for 48 h at 60°C to promote polymerization. Using an ultramicrotome (Leica EMUC6;), the epoxy blocks were sliced into 100 nm-thin slices, and the sections were arranged in copper grids. The slices were stained with uranyl acetate and lead citrate (FEI Tecnai BioTWIN;) and examined under TEM.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism (version 6.0;). Data were analyzed using one-way ANOVA and the Tukey post-hoc test. P-values of ≤0.05 were considered statistically significant.

■ RESULTS

MTT Assay

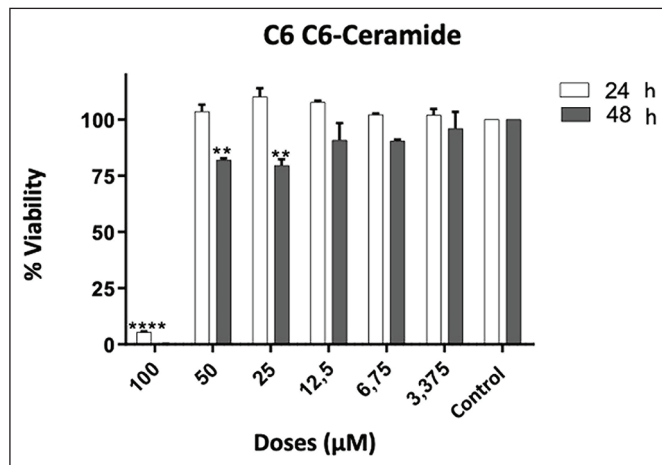The effect of C6 ceramide on the viability of C6 glioma cells

Figure 1: Growth inhibitory effects of different concentrations of C6-Ceramide on C6 glioma cells after 24 and 48 hours of application using MTT assay. p values less than 0.05 were accepted as statistically significantly different and are shown with asterisk (*). (**** indicates p<0,0001; ** indicates p<0,001).

was dose- and time-dependent. The maximum lethal effect was observed with 100 µM C6 ceramide application after 24 h and 48 h. Additionally, there was a statistically significant decline in cell viability (p<0.0001). In comparison with the controls, application of 25 and 50 µM C6 ceramide led to an increase in cell viability in 24 h, followed by a decrease in cell viability in 48 h. The decrease after 48 h was statistically significant (p<0.001). There was a statistically significant decrease in cell viability in 24 h after application of 100 µM C6 ceramide (p<0.013) (Figure 1). The effects of C6 ceramide on the survival of C6 glioma cells depended on the dose and passage of time. The half maximal inhibition concentrations of C6 ceramide after 24 and 48 h were 77 µM and 69 µM, respectively.

Confocal Microscopy Findings

Figure 2A shows the morphological structure of the C6 rat glioma cells in the control group following double-staining with phalloidin and acridine orange. Figure 2B and 2C show the morphological structure of the C6 glioma cells 2 h after C6 ceramide application. The C6 control cells remained unchanged, with a compact morphology (Figure 2A). C6 ceramide application produced chromatin condensation, cytoskeleton fragmentation, and holes in the cytoskeleton (Figures 2B and 2C).

Transmission Electron Microscopy Findings

The ultrastructure of the control C6 cells remained unchanged and compact (Figure 3). Application of IC_{50} inhibition concentrations of C6 ceramide produced membrane blebbing (Figure 4), a characteristic change during the process of apoptosis. Membrane blebbing is the outward bulging or protrusion of the cell's plasma membrane. These protrusions frequently take the form of tiny, rounded, and amorphous vesicles or blebs on the cell surface. Membrane blebbing is a dynamic phenomenon that is linked to cytoskeleton reorganization and modifications in membrane integrity. Its presence indicates apoptotic cell death.

C6 ceramide application also caused a loss of the mitochondrial cristae; the normally well-defined and highly folded inner mitochondrial membrane appeared damaged or altered,
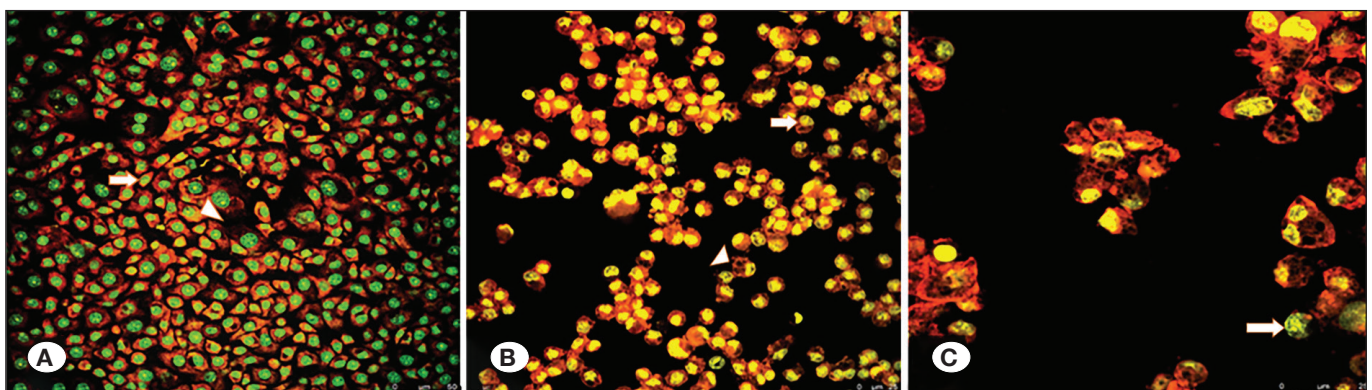

Figure 2: Confocal micrographs of C6 control cells **(A)** and C6 ceramide treated C6 cells **(B and C)** following double staining with acridine orange and phalloidin. **A:** Arrow-compact nucleus, Arrowhead-Compact cytoskeleton. **B:** Arrow-chromatin condensation, Arrowhead-disintegration of cytoskeleton as fragmentations, **C:** Arrowhead-Chromatin condensation and nuclear shrinkage.
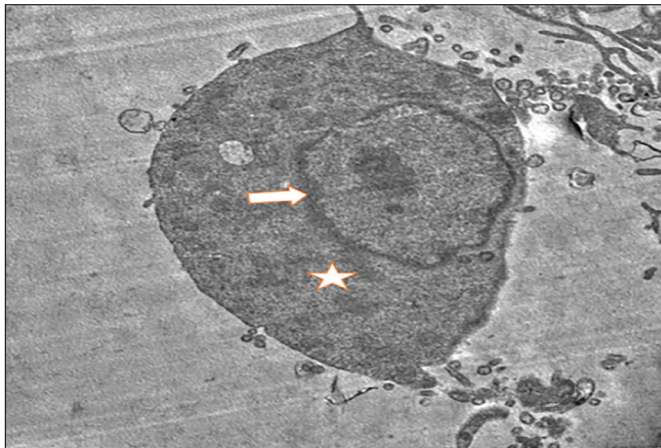
Figure 3: Transmission electron micrograph of control C6 cells. **Asterisk:** cytoskeleton and organelles, **Arrow:** normal nucleus membrane.
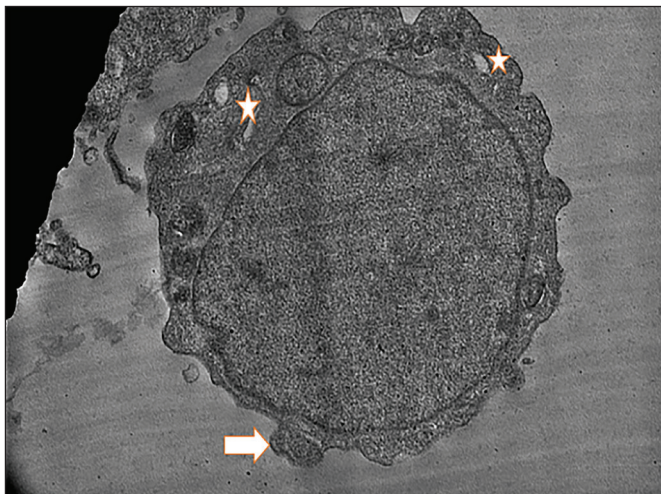

Figure 4: Transmission electron microscopic image of C6 cells treated with IC_{50} inhibition concentration of C6 ceramide for 24 h. Arrow: membrane blebbing, Asterisk: Loss of cristae of mitochondrion.

and sometimes even disappearing altogether. This structural change is often associated with mitochondrial dysfunction and is indicative of cellular stress or damage to the mitochondria.

■ DISCUSSION

In this study, we evaluated the ability of C6 ceramide to induce apoptosis by identifying morphological and ultrastructural alterations under confocal microscopy and TEM. Using the MTT assay, the proapoptotic impact of C6 ceramide on human C6 glioma cells was evaluated. Our results demonstrated that C6 successfully encouraged apoptosis and that its cytotoxic effects led to changes in the ultrastructure and morphology of C6 glioma cells.

Ceramides are lipid messengers in nerve cells, causing cell death and inhibition of cancer cell proliferation when present

endogenously or exogenously in the cell at high levels. Thus, ceramides are considered to be useful targets for cancer therapy. Numerous papers underline the apoptotic effects of ceramides, especially C2 and C6, when administered to the cell exogenously (5,7). C6 ceramide in particular can cross the BBB (1). Thus, it is considered to be a promising therapeutic agent for brain cancer(1). Our cytotoxicity assay demonstrated that the short-term application (24 hours and 48 hours) of C6 ceramide effectively exerted an antiproliferative effect on the C6 rat glioma cells in a dose- and time-dependent manner.

Sphingolipids play a crucial role in cell growth and apoptosis. Thus, they are important targets in cancer therapy. In prostate cancer cell lines, amplification of acid ceramidase (aCDase), an enzyme that degrades ceramide and is implicated in tumor genesis, causes an increase in cellular resistance to C6 ceramides. Additionally, overexpression of aCDase causes a decrease in intracellular ceramides and inhibition of cellular proliferation in other cancers, including head and neck cancer, breast cancer, and melanoma (15,17,18,21). This resistance to C6 ceramides leads to apoptosis resistance (14). Thus, inhibition of aCDase may increase the intracellular ceramide levels, which will induce apoptosis (15). N-oleoylethanolamine reportedly decreases aCDase activity and induces radiation-triggered apoptosis in human glioma cells (6). In our study, exogenous application of C6 ceramide to rat glioma C6 cells effectively altered the cellular morphology and ultrastructure. It caused the cells to shrink and become circular. Shrinkage of the nuclei, chromatin condensation, and cytoskeleton holes, which are characteristic findings of apoptosis, were detected in the C6 rat glioma cells treated with ceramide C6.

In cancer therapy, sphingolipid-based therapies are considered to be promising, and ceramide analogs that induce apoptosis are preferred. Evaluation of human breast cancer MDA-MB-231 cells has demonstrated that liposomal C6 ceramide induces apoptosis (2,20). Furthermore, tumor growth was inhibited by the application of the pegylated liposomal form of C6 ceramide to BALB/c breast adenocarcinoma mouse models(19). In one study, the chronic myeloid leukemia cell line K562 underwent apoptosis via the caspase-8 and JNK pathways when exogenously administered C6 ceramide (12). This finding was similar to that of our study. In our study, the C6 rat glioma cell line was used to assess the C6 ceramide's cytotoxic, antiproliferative, and proapoptotic effects. Our findings indicated that C6 ceramide hindered the C6 cells' capacity to survive and grow in a dose- and time-dependent manner.

Banerjee et al. was the first to demonstrate that ceramides can induce apoptotic cell death in astrocytic tumors using the caspase3 ELISA test (1). In our study, we demonstrated apoptosis using the caspase3 ELISA test as well as confocal microscopy and TEM. Our study findings indicate that increasing the intracellular ceramide levels is an option for cancer therapy. The unconventionally high ceramide levels in C6 cells promoted cell death. Thus, exogenous application of ceramides, drug-induced synthesis of ceramide, or inhibition of ceramide hydrolysis may be prospective therapeutic options for cancer.

CONCLUSION

Our study findings provide critical understanding of the effects of C6 ceramide on C6 glioma cells, especially its strong cytotoxic effect. C6 ceramide produced a dose- and time-dependent decrease in cell viability, with a statistically significant lethal effect at a concentration of 100 µM after 24 h and 48 h. These results were complemented by compelling findings on confocal microscopy and TEM, which demonstrated distinct morphological and ultrastructural changes induced by C6 ceramide. These findings further highlighted C6 ceramide's profound impact on C6 glioma cells. Collectively, our study findings deepen our understanding of ceramide's potential therapeutic role in glioma-related diseases and emphasize its potential as a cytotoxic agent worthy of continued investigation.

Declarations

Availability of data and materials: The datasets generated and/or analyzed during the current study are available from the corresponding author by reasonable request.

Disclosure: Authors declare no conflict of interest.

AUTHORSHIP CONTRIBUTION

Study conception and design: MB, HMK

Data collection: MB, HMK, EY, CVS, GK

Analysis and interpretation of results: MB, HMK

Draft manuscript preparation: EY, CVS

All authors (MB, CVS, GK, EY, HMK) reviewed the results and approved the final version of the manuscript.

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