Pivotal Role of the Cell Death Factor BNIP3 in Ceramide-Induced Autophagic Cell Death in Malignant Glioma Cells

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INTRODUCTION

Malignant gliomas are among the most devastating cancers (1). These tumors rapidly grow and invade into the surrounding brain parenchyma. Therefore, it is often impossible to achieve complete surgical resection without severe neurological damages. Despite advances in the diagnosis and treatment, the prognosis of patients with malignant gliomas remains very poor. Indeed, we need to explore a new approach for treatment of malignant gliomas. It has been shown recently that ceramide, the basic unit of the lipid sphingomyelin, is inversely associated with poor prognosis of malignant gliomas (2). Although the potential benefits of ceramide-based therapy are proposed, the mechanism by which ceramide exhibits the antitumor effect on malignant glioma cells remains to be elucidated.

Ceramide is produced in cells upon hydrolysis of sphingomyelin or by de novo synthesis (3). When sphingomyelinase is activated to cleave the bond between ceramide and the phosphoric acid of sphingomyelin, ceramide is produced. Ceramide synthase also produces ceramide de novo through N-acetylation of sphinganine and the addition of a double bond. Ceramide is produced by diverse stress stimuli including chemotherapy or γ-irradiation (4, 5). To date, ceramide has been recognized as an important second messenger molecule involved in the signaling pathways that control cell proliferation, differentiation, and death (6, 7). In particular, ceramide-mediated apoptotic cell death is extensively investigated (8–10). On the other hand, several investigations indicate that nonapoptotic cell death is induced in certain tumor or normal cells by ceramide (11–13). However, the molecular pathways underlying the latter cell death remain poorly understood.

In the present study, we provide evidence that ceramide induces autophagic cell death in malignant glioma cells. Autophagy is a term used to describe the process of protein recycling, typically observed in hepatocytes after amino acid deprivation (14). More attention has been paid recently to autophagy as one of the defensive mechanisms for cancer cells (15, 16). By inducing autophagy, cancer cells recycle molecules for biosynthetic or metabolic reactions and subsequently adapt to adverse conditions after anticancer therapy. This autophagy is basically reversible and provides a mechanism of self-defense for cancer cells. Radiation (15) or chemotherapeutic agent temozolomide (17) induces defensive autophagy in cancer cells. In several cases, however, autophagy plays a central role in the elimination of cancer cells by triggering a nonapoptotic cell death program (18, 19). This type of autophagy is irreversible and is designated as type II programmed cell death or autophagic cell death, in contrast to apoptosis, which is referred to type I programmed cell death (16, 20). Anticancer agents such as tamoxifen or arsenic trioxide induce destructive autophagy or autophagic cell death in cancer cells (18, 19). It is quite possible that autophagy in general is a reflection of cellular stress rather than the cause of it. When the damage is benign, autophagy, by encapsulating dying organelles and providing amino acids, may enable the cells to survive stress. When the damage is too severe, autophagy may slow cell death but cannot prevent it. To date, however, very little is known about the molecular pathways that regulate autophagy in cancer. We demonstrate here that the cell death-inducing mitochondrial protein, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3; Ref. 21), plays a central role in ceramide-induced autophagic cell death in malignant glioma cells. To our knowledge, this is the first report adding a new concept to ceramide-induced cell death pathways.

MATERIALS AND METHODS

Reagents. C2-ceramide (N-acetyl-e-erythro-sphingosine; Calbiochem Co., La Jolla, CA) was prepared as a 50 mM stock solution in DMSO (Sigma Chemical Co., St. Louis, MO). Final concentration of DMSO in culture medium is <0.2% volume. DNA-binding Hoechst 33258 and acridine orange were purchased from Sigma.

Antibodies. Anticeramide-deacetylase protein 1 light chain 3 (LC3) antibody was kindly provided by Drs. Noboru Mizushima and Tamotsu Yoshimori (National Institute for Basic Biology, Okazaki, Japan). Anti-β-actin and anti-β-galactosidase (β-gal) antibodies were purchased from Sigma and Promega (Madison, WI), respectively. Anti-BNIP3 antibody was purchased from BD Biosciences Pharmingen (San Diego, CA).

Tumor Cell Lines. Human malignant glioma U373-MG and T98G cells were purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in D–MEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 4 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO2.

Cell Proliferation Assay. The cytotoxic effect of C2-ceramide on malignant glioma cell lines was determined by using a trypan blue dye exclusion...
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Fig. 1. Effect of ceramide on malignant glioma cells. A, cytotoxic effect of C2-ceramide in U373-MG and T98G cells. After exposure to C2-ceramide or DMSO alone for 24 h, the cells were trypsinized, and the number of viable cells was counted. The cytotoxic effect of C2-ceramide was dose-dependent. □, C2-ceramide (+); ■, C2-ceramide (-). Results shown are the means of three independent experiments; bars, ±SD. B, rate of trypan blue-positive cells in attached tumor cells treated with C2-ceramide. After exposure to C2-ceramide (25 μM), the rate of trypan blue-positive cells was counted. The incidence of cell death was increased in a time-dependent manner. Results shown are the means of three independent experiments; bars, ±SD. C, effect of C2-ceramide on cell cycle. U373-MG (a and c) and T98G cells (b and d) treated with or without C2-ceramide (25 μM) for 24 h were analyzed for cell cycle. The population in G2-M phase increased in C2-ceramide-treated tumor cells (c and d) compared with the control (a and b). The population in sub-G1 phase in treated cells was very low. D, rate of apoptotic cells in tumor cells treated with C2-ceramide. After exposure to 25 μM C2-ceramide for up to 24 h, the rate of apoptotic cells was counted. The rates of apoptotic cells in attached or detached tumor cells treated with 25 μM C2-ceramide were <4% or 8% over a 24-h incubation, respectively. E, effect of C2-ceramide on apoptosis in malignant glioma cells. Nuclei were stained with Hoechst 33258 to detect apoptotic cells in tumor cells treated with C2-ceramide. After exposure to 25 μM C2-ceramide for up to 24 h, the rate of apoptotic cells was counted. No significant apoptotic cells were observed in attached or detached tumor cells treated with C2-ceramide for 24 h. Bars, 50 μm. Results shown are the means of three independent experiments; bars, ±SD.

assay as described previously (17, 19). Tumor cells (2 × 10^6 cells/well) were seeded in 96-well flat-bottomed plates. After 24 h, tumor cells were fed with culture medium containing 0.1% fetal bovine serum and treated with C2-ceramide at concentrations ranging from 0 to 100 μM for up to 24 h. Then cells were detached by trypsinization, and the number of viable cells was counted.

Cell Cycle Analysis. For cell cycle analysis, treated tumor cells were trypsinized, fixed with 70% ethanol, and stained with propidium iodide by using the Cellular DNA Flow Cytometric Analysis Reagent set (Roche, Indianapolis, IN) as described previously (17, 19). Samples were analyzed for DNA content with the FACSscan using CellQuest software (Becton Dickinson, San Jose, CA).

Hoechst DNA Staining. Nuclei were stained with Hoechst 33258 to detect chromatin condensation or nuclear fragmentation characteristic of apoptosis as described previously (22). Treated tumor cells were fixed with 4% paraformaldehyde and stained with Hoechst 33258 (0.5 μg/ml) for 15 min. To determine whether detached tumor cells exhibit apoptotic features, detached cells were collected after a 24-h treatment, fixed, and deposited on slide glasses by centrifugation of 1200 rpm for 1 min with Cytospin Cytocentrifuge (Shandon Inc., Pittsburgh, PA) before Hoechst 33258 staining. Two-hundred cells were counted and scored for the incidence of apoptotic chromatin changes under a fluorescence microscope. The green fluorescent protein (GFP)-tagged LC3 expressing vector kindly provided by Drs. N. Mizushima and T. Yoshimori, involvement of LC3 in tumor cells treated with C2-ceramide at a concentration of 25 μM for 24 h, fixed for 2 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% OsO4 in the same buffer, then subjected to the electron microscopical analysis as described previously (17, 19). Representative areas were chosen for ultra-thin sectioning and viewed with a Hitachi 7600 electron microscope.

Detection and Quantification of Acidic Vesicular Organelles (AVO) with Acidine Orange Staining. Autophagy is the process of sequestering cytoplasmic proteins into the lytic component and characterized by development of AVOs. To detect and quantify AVOs in C2-ceramide-treated cells, we performed the vital staining with acidine orange as described previously (15, 17, 19). Treated tumor cells were stained with acidine orange, adding at a final concentration of 1 μg/ml for 15 min. Samples were examined under a fluorescence microscope. To quantify the development of AVOs, cells were stained with acidine orange for 15 min, detached by trypsinization, and collected for the FACSscan using CellQuest software.

Involvement of Microtubule-Associated Protein LC3. LC3, a mammalian homologue of Apg8p, is recruited to the autophagosome membrane during autophagy (23). The green fluorescent protein (GFP)-tagged LC3 expressing cells were used recently to demonstrate induction of autophagy (17, 24, 25). GFP-LC3 cells presented a diffuse distribution under control, whereas a punctate pattern of GFP-LC3 expression was increased in number and fluorescence intensity by autophagy. Therefore, using the GFP-LC3 expression vector kindly provided by Drs. N. Mizushima and T. Yoshimori, involvement of LC3 in tumor cells treated with C2-ceramide was determined. Tumor cells were transfected with the GFP-LC3 expression vector using FuGENE 6.
Transfection Reagent (Roche). After overnight culture, cells were treated with or without C2-ceramide, fixed with 4% paraformaldehyde, and examined under a fluorescence microscope.

**Western Blotting.** Soluble protein for Western blotting was harvested from treated tumor cells lysed in extraction buffer as described previously (22). Equal amounts of protein estimated by the Bio-Rad Protein Assay were separated by SDS-PAGE (18% or 8–16% gel; Bio-Rad, Richmond, CA) and transferred to a Hybond-P membrane (Amersham Co., Piscataway, NJ). The membrane was subjected to Western blotting using an ECL chemiluminescence reagent (Amersham).

**Flow Cytometric Analysis of Mitochondrial Membrane Potential.** Rhodamine 123 (Molecular Probe Inc., Eugene, OR), a cationic voltage-sensitive probe, was used to detect changes in mitochondrial membrane potential by using flow cytometry as described previously (26). Treated tumor cells were collected with trypsinization, washed twice with PBS, and stained with 1 μM rhodamine 123 in PBS in the dark at 37°C for 1 h. Samples were washed, resuspended with PBS, and then analyzed with the FACScan using CellQuest software.

**Reporter Assay.** The transcriptional activities were determined by the luciferase expression assay as described previously (27). The BNIP3 promoter-luciferase reporter plasmid and the mutation of the BNIP3 promoter (site-directed mutation located at 234 bp upstream of BNIP3 translation start codon; Ref. 28) were kindly provided by Dr. Richard K. Bruick (University of Texas Southwestern Medical Center, Dallas, TX). Tumor cells were plated at a density of 5 × 10^3 cells/ml and cotransfected with the above luciferase reporter plasmid and pRL-SV40 (Promega; 0.2 μg each) using FuGENE 6 Transfection Reagent. The cells were incubated for 24 h, then treated with C2-ceramide for 24 h and analyzed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s protocol.

**Semiquantitative Reverse Transcription-PCR.** The expression of BNIP3 mRNA in treated tumor cells was analyzed by semiquantitative reverse transcription-PCR amplification as described previously (27). Total RNA was isolated from treated cells using the RNeasy kit (Qiagen Inc., Valencia, CA). Equal amount of RNA was reverse-transcribed to cDNA and amplified by PCR with ThermoScript Reverse Transcription-PCR System and Platinum Taq DNA polymerase (Invitrogen). PCR was performed with gene-specific primers for 21–30 cycles at 94°C for denaturing, 55°C for annealing, and 72°C for extension as described previously (29). Glyceradehyde-3-phosphate dehydrogenase was used as an internal control.

**Transient Transfection with BNIP3 Expression Vector.** Tumor cells were cotransfected with GFP-LC3 plasmid and the expression vector of BNIP3 (Ref. 30; kindly provided by Dr. A. H. Greenberg, University of Manitoba, Winnipeg, Manitoba, Canada) using FuGENE 6 Transfection Reagent. As a control, a β-gal expression vector was used. After incubation for 48 h after the transfection, cells were fixed with 4% paraformaldehyde, blocked with 1% BSA, and incubated overnight at 4°C with anti-BNIP3 or β-gal antibody at a 1:100 dilution with 1% BSA. After incubation for 1 h at room temperature with secondary antibody (Vector Lab. Inc., Burlingame, CA), samples were visualized with Texas red avidin D (Vector Lab. Inc.) and examined under a fluorescence microscope.

**Statistical Analysis.** All of the experiments were repeated at least three times. The data were expressed as means ± SD. Statistical analysis was performed by using Student’s t test (two-tailed). The criterion for statistical significance was taken as P < 0.05.

**RESULTS**

**Cytotoxic Effect of C2-Ceramide on Malignant Glioma Cells.** Tumor cells were treated with C2-ceramide at concentrations ranging from 0 to 100 μM or with DMSO alone for 24 h, and the number of viable cells was determined. As shown in Fig. 1A, treatment with C2-ceramide inhibited the proliferation of U373-MG and T98G cells in a dose-dependent manner. With 25 μM C2-ceramide, the viable cell number of treated tumor cells decreased below the initial cell number (1.95 × 10^4 for U373-MG cells and 2.78 × 10^4 for T98G cells) at the time of addition of C2-ceramide, suggesting induction of cell death. To determine what fraction of the attached cell population was dead by C2-ceramide, we counted the rate of trypan blue-positive cells. As shown in Fig. 1B, the incidence of cell death was increased in a time-dependent manner. Eighteen percent of U373-MG cells and 9% of T98G cells attached on the plates were dead 24 h after exposure to 25 μM C2-ceramide. On the basis of these results, we decided to use C2-ceramide at a concentration of 25 μM for subsequent studies.

**C2-Ceramide Induces Nonapoptotic Cell Death and G2-M Arrest in Malignant Glioma Cells.** To examine whether C2-ceramide affects cell cycle and induces apoptosis, we performed the DNA flow-cytometric analysis. As shown in Fig. 1C, treatment with 25 μM C2-ceramide for 24 h decreased the cell population at the G1 phase and increased the population at the G2-M in U373-MG and T98G cells. The population in sub-G1 phase characteristic of apoptosis was increased from 0.32% to 1.93% (6-fold) in U373-MG cells and from 0.55% to 2.29% (4-fold) in T98G cells, but the percentage still remained very low. Additionally, nuclei of tumor cells treated with or without 25 μM C2-ceramide for 24 h were stained with Hoechst 33258 to determine induction of apoptosis morphologically. No significant number of cells exhibiting apoptotic morphology such as chromatin condensation or nuclear fragmentation was detected in attached or detached U373-MG and T98G cells treated with 25 μM C2-ceramide for 24 h (Fig. 1E). Treatment with cisplatin (5 μg/ml) for 72 h induced apoptosis in 16% of U373-MG cells and 22% of T98G cells. Without C2-ceramide, the rates of apoptotic cells of U373-MG and T98G were <4% (Fig. 1D). On the other hand, the rates of apoptosis in attached tumor cells treated with 25 μM C2-ceramide for up to 24 h were also <4%. In addition, the rates of apoptotic cells in tumor cells detached...
C₂-ceramide (-) 24 hr

C₂-ceramide (+) 24 hr

Fig. 2. Electron micrographs showing the ultrastructure of U373-MG cells treated with C₂-ceramide (25 μM) for 24 h. A, control; very few autophagic vacuoles were observed in U373-MG cells treated without C₂-ceramide for 24 h. B, C₂-ceramide-treated U373-MG cells. Numerous autophagic vacuoles (arrows) and empty vacuoles (arrowheads) were observed. N, nucleus. Bars (A and B), 1.0 μm.

by treatment with 25 μM C₂-ceramide for 24 h were <8%, and there was no significant increase in apoptotic fraction (Fig. 1D). These results indicate that C₂-ceramide induces G₂-M arrest in U373-MG and T98G cells, and a large fraction of treated tumor cells die by nonapoptotic cell death within 24 h after the treatment.

C₂-Ceramide Induces Autophagy in Malignant Glioma Cells. It has been demonstrated recently that nonapoptotic autophagy is detected in a variety of cancer cells treated with radiation or chemotherapy (15, 17–19). Therefore, we investigated whether C₂-ceramide induces autophagy in malignant glioma cells using the electron microscopic analysis. As shown in Fig. 2B, numerous autophagic vacuoles (arrows) and empty vacuoles (arrowheads) were observed in U373-MG cells treated with 25 μM C₂-ceramide for 24 h. Most of the autophagic vacuoles contained intact cytoplasmic structure, lamellar structure, or residual digested materials. However, there was no chromatin condensation or fragmentation characteristic of apoptosis. On the other hand, tumor cells treated with DMSO alone for 24 h exhibited few autophagic features (Fig. 2A). Then we counted the number of cell profiles on the ultra-thin sections that contain >10 autophagic vacuoles. Of 121 U373-MG cells, 121 treated with 25 μM C₂-ceramide for 24 h had such profiles (100%), whereas only 1 of 121 tumor cells treated with DMSO alone for 24 h was autophagic (<1%). These results indicate that U373-MG cells treated with 25 μM C₂-ceramide for 24 h are highly active in autophagic process.

Development of AVOs in Malignant Glioma Cells Treated with C₂-Ceramide. Autophagy is characterized by AVO development, which is detected and measured by vital staining of acridine orange. As shown in Fig. 3A, vital staining of U373-MG cells with acridine orange showed development of AVOs in a time-dependent manner. To quantify the fractional volume and/or acidity of AVO in tumor cells treated with C₂-ceramide, we performed a flow cytometric analysis. Treatment with 25 μM C₂-ceramide for 24 h increased the percentage of cells that have prominent red fluorescence from 5.11% to 25.88% in U373-MG cells and from 5.06% to 30.49% in T98G cells (Fig. 3B). These results indicate that C₂-ceramide induces development of AVOs in malignant glioma cells.

Involvement of LC3 in C₂-Ceramide-Induced Autophagy. LC3 is localized in autophagosome membranes during amino acid starvation-induced autophagy (23, 24). To assess whether LC3 is involved in C₂-ceramide-induced autophagy, we transfected tumor cells with the GFP-LC3 expression vector. As shown in Fig. 4A, panel a, untreated U373-MG cells showed diffuse distribution of green fluorescence. On the other hand, treatment with 25 μM C₂-ceramide increased cells with a punctate pattern of GFP, representing autophagic vacuoles in a time-dependent manner (Fig. 4A, panels b–d). The rate of cells with GFP-LC3 dots was 2.5% (0 h treatment), 3.0% (1 h), 6.1% (5 h), and 24.2% (24 h; Fig. 4B) in U373-MG cells treated with C₂-ceramide. However, only 2.6% of U373-MG cells treated without C₂-ceramide for 24 h expressed GFP-LC3 dots. Similar results were observed in T98G cells (data not shown). Recent investigation shows that there are two forms of the LC3 proteins in cells: LC3-I and LC3-II (24). LC3-I is the cytoplasmic form of LC3 and is processed into LC3-II, which is associated with the autophagosome membrane. Therefore, the amount of LC3-II is correlated with the extent of autophagosome formation. Using the immunoblotting analysis with anti-LC3 antibody, we examined the expressions of LC3-I (18 kDa) and LC3-II (16 kDa) in malignant glioma cells treated with C₂-ceramide. As shown in Fig. 4C, the expression of total LC3 (LC3-I and LC3-II proteins) increased in U373-MG and T98G cells 24 h after exposure to C₂-ceramide, suggesting that LC3 is newly synthesized by C₂-ceramide. Moreover, a marked increase in LC3-II protein was detected in both tumor cells after a 24-h treatment with C₂-ceramide. These results indicate that LC3 is involved in C₂-ceramide-induced autophagy, and C₂-ceramide stimulates not only the synthesis of LC3 protein, but also the conversion of a fraction of LC3-I into LC3-II.

C₂-Ceramide Decreases the Mitochondrial Membrane Potential. C₂-ceramide has been reported to induce apoptotic cell death in several cancer cells via mitochondrial disruption (31, 32). Therefore, to determine whether mitochondrial membrane integrity is damaged in autophagic cell death induced by C₂-ceramide, mitochondrial membrane potential was measured using rhodamine 123. As shown in Fig. 5, treatment with 25 μM C₂-ceramide for 24 h induced loss of membrane potential in U373-MG and T98G cells. These results indicate that C₂-ceramide-induced autophagic cell death is associated with mitochondrial dysfunction.

C₂-Ceramide-Induced Autophagic Cell Death Is Associated with BNIP3. BNIP3 is one of the mitochondria-associated cell death proteins and induces nonapoptotic cell death through mitochondrial...
dysfunction by opening the permeability transition (30). Therefore, it is possible that BNIP3 is involved in C₂-ceramide-induced autophagy in malignant glioma cells. To test this possibility, we first examined whether C₂-ceramide activates BNIP3 gene transcriptionally using the BNIP3 promoter-luciferase reporter construct. As shown in Fig. 6A, the luciferase activity of BNIP3 promoter was increased 3.3-fold in U373-MG cells and 2.1-fold in T98G cells after treatment with 25 μM C₂-ceramide for 24 h compared with the control (without C₂-ceramide for 24 h). An increase in transcriptional activity of BNIP3 gene in U373-MG or T98G cells treated with C₂-ceramide was significant when compared with the BNIP3 mutant promoter (P<0.01). Furthermore, treatment with 25 μM C₂-ceramide increased the mRNA expression of BNIP3 gene in U373-MG cells and T98G cells in a time-dependent manner (Fig. 6B). In addition, C₂-ceramide increased the expression of the dimer but not the monomer form of BNIP3 protein in U373-MG cells (Fig. 6C). Most of the Bcl-2 family proteins such as BNIP3 work with dimer formation (21). Therefore, an increase in the dimer expression of BNIP3 protein is supposed to correlate with an increase in their activities. These results indicate that C₂-ceramide-induced autophagic cell death is associated with activation of BNIP3 gene.

Exogenous Expression of BNIP3 Induces Autophagy. On the basis of the above results, we speculated that C₂-ceramide activated BNIP3 gene and subsequently induced autophagy in U373-MG and T98G cells. To examine this speculation, we determined whether...
exogenous expression of BNIP3 protein induces autophagy in tumor cells without C2-ceramide treatment. To detect autophagic cells after transfection of U373-MG or T98G cells with BNIP3 expression vector, the GFP-LC3 construct was cotransfected. As shown in Fig. 7A, BNIP3-positive cells (Fig. 7A, panel d) exhibited autophagic change with a punctate pattern of GFP-LC3 (Fig. 7A, panel b) 48 h after BNIP3 gene transfection. In contrast, most of the β-gal-positive cells (Fig. 7A, panel c) did not show autophagic pattern of GFP-LC3 expression (Fig. 7A, panel a). To quantify these findings, we counted 200 BNIP3- or β-gal-positive tumor cells and scored the percentage of autophagy. As shown in Fig. 7B, when the control β-gal gene was transduced, 3.3% of U373-MG cells and 6.7% of T98G cells showed autophagy. Overexpression of BNIP3 induced autophagy in 10.3% of U373-MG cells and 24.8% of T98G cells. The ability of BNIP3 to induce autophagy was significantly higher than that of β-gal gene (P < 0.01). These results indicate that BNIP3 induces autophagy in malignant glioma cells without C2-ceramide treatment. Taken together, we conclude that BNIP3 plays a central role in C2-ceramide-induced autophagy.

**DISCUSSION**

In the present study, we have demonstrated that C2-ceramide induces nonapoptotic, autophagic cell death in malignant glioma cells. The inability of C2-ceramide to induce apoptosis in U373-MG or T98G cells is not due to a defective apoptotic pathway, because apoptosis was detected in these tumor cells treated with the chemotherapeutic agent, cisplatin (22). LC3, which is recruited in autophagy induced by amino acid deprivation, is also recruited by C2-ceramide. LC3 was identified as a protein that copurified with microtubule-associated protein 1A and 1B from brain samples (33). It has been demonstrated recently that LC3 is the first mammalian protein that specifically associates with autophagosome membrane (23). Just before or after the completion of autophagosome formation, the majority of LC3 remains in the autophagosomal membrane. Therefore, LC3 is regarded as a specific marker to detect autophagosome formation (17, 23–25). We additionally provide for the first time direct evidence that mitochondria-associated cell death protein, BNIP3, plays a particularly important role in C2-ceramide-induced autophagic cell death. Initially, BNIP3 was identified as an adenovirus E1B 19 kDa-interacting protein (34). BNIP3 possesses the Bcl-2 homology 3 domain, which is a short stretch of sequences shared by all of the Bcl-2 family proteins (both the antiapoptotic and proapoptotic members). Overexpression of BNIP3 induces caspase-independent nonapoptotic or necrosis-like cell death (30, 35). BNIP3-induced cell death is accompanied by rapid and profound mitochondrial dysfunction (30), which is consistent with our results. Of U373-MG cells treated with C2-ceramide for 24 h, 24% exhibited autophagic GFP-LC3 dots, whereas...
overexpression of BNIP3 induced GFP-LC3 dots in only 10% of tumor cells. These differences might be related to transfection efficiency of BNIP3 or to the possibility that ceramide affects cell death by modulating additional pathway. Additional study is necessary to understand the discrepancy. Recent investigations show that BNIP3 (35–37) or ceramide (38) is up-regulated in hypoxia-mediated cell death. In this study, we used the BNIP3 promoter containing site-directed mutation in putative hypoxia-inducible factor-1α-responsive element (28) as a negative control. The fact that mutation of the hypoxia-inducible factor-1α-responsive element led to the loss of C2-ceramide-mediated responsiveness in U373-MG and T98G cells raises the possibility that C2-ceramide may induce hypoxic stress status in tumor cells, and subsequently BNIP3 is activated, leading to autophagic cell death. This possibility may be supported by a more recent report demonstrating that hypoxia induces nonapoptotic cell death in malignant glioma cells (39).

Ceramide-induced cell death is mediated by at least two pathways: transcription-dependent and -independent pathways (3). The transcription-dependent pathway is caused by the interaction of members of the tumor necrosis factor superfamily of receptors and their specific ligands (7). It is thought that this type of transcription-dependent ceramide signaling is correlated with the sensitivity to chemotherapeutic agents (40). On the other hand, the transcription-independent pathway is mediated by stress stimuli such as radiation (41) and is shown to affect apoptosis-related proteins of the Bcl-2 family (42). By altering the association between proapoptotic and antiapoptotic members of the Bcl-2 family, ceramide initiates caspase-dependent cell death in leukemia cells with a loss of mitochondrial membrane integrity and the release of proapoptotic molecules including cytochrome c into the cytoplasm (43). However, the ceramide cell death pathway is a complex system of signal reinforcement and magnification that has not been fully understood. Several investigations demonstrate recently that ceramide induces nonapoptotic cell death without caspase activation (11–13). Mochizuki et al. (13) clearly showed that ceramide-induced nonapoptotic cell death in malignant glioma cells is inhibited by the activation of Akt/protein kinase B pathway. A phosphatidylinositol 3′-kinase signaling pathway that is located upstream of Akt is involved in autophagic process (44). Moreover, the signaling of autophagy is regulated in some tumor cells by the activity of tumor suppressor genes such as Beclin-1 and PTEN (45, 46). As other factors controlling autophagy, mammalian target of rapamycin, the p70S6 kinase, and the eukaryotic initiation factor-2α kinase have been reported recently (47, 48). Additionally, the death-associated protein kinase and death-associated protein kinase-related protein kinase are involved in the formation of autophagic vacuoles (49). Therefore, induction of nonapoptotic autophagic cell death may depend on the balance between the input of mitochondria-associated cell death signals and the activation of the above signaling pathways including phosphatidylinositol 3′-kinase/Akt/mammalian target of rapamycin.

In summary, we have demonstrated that C2-ceramide induces autophagic cell death in malignant glioma cells. Mitochondria-associated cell death protein BNIP3 plays a central role in the cell death. These findings not only add a novel concept to ceramide-induced cell death pathways but also suggest a potential of ceramide-based therapy for malignant gliomas.

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