Enhancement of C2-ceramide antitumor activity by small interfering RNA on X chromosome–linked inhibitor of apoptosis protein in resistant human glioma cells

MANABU HATANO, M.D., MASAAMI MIZUNO, M.D., PH.D., AND JUN YOSHIDA, M.D., PH.D.
Departments of Neurosurgery and Molecular Neurosurgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

Objective. Many human glioma cells are resistant to ceramide. In this study the authors investigated the mechanisms of that resistance and considered ways to overcome it.

Methods. The authors first administered C2-ceramide (N-acetylphosphosine) to human glioma cells from rare cell lines susceptible to C2-ceramide (SKMG1 and U87MG) and other cell lines resistant to it (U251SP, T98G, SKAO2, and U251MG). Following this, the authors analyzed the statuses of transduction signals such as cell viability, morphological changes, caspases, mitochondrial membrane potential, apoptosis-inducing factor, oligonucleosomal DNA fragmentation, and the inhibitor of apoptosis protein (IAP) family.

Conclusions. Ceramide resistance was found to arise from the inhibition of caspase-7 induced by IAPs, especially X chromosome–linked IAP (XIAP). Small interfering RNA (siRNA) on XIAP quenched that resistance in ceramide-resistant human glioma cells (U251SP, T98G, SKAO2, U251MG), indicating that a siRNA for XIAP may be a useful tool for overcoming the resistance to ceramide in human glioma cells.

Key Words • ceramide • X chromosome–linked inhibitor of apoptosis protein • small interfering RNA • glioma • apoptosis • mitochondria

Ceramide, a second messenger of the sphingomyelin pathway, regulates a number of cellular processes such as apoptosis and cell-cycle arrest in various cell types. Especially in hematopoietic cells, such as those of acute myeloid leukemia, ceramide has been found to exhibit a high therapeutic efficacy through apoptosis. Gliomas are no exception to this regulation. Sanchez and associates reported that selective activation of the CB2 receptor signaled apoptosis via de novo enhanced ceramide synthesis in gliomas. Riboni, et al., support the rationale for the potential benefits of a ceramide-based chemotherapy in patients harboring glioma tumors, because a variety of anticancer drugs, such as Taxol and adriamycin, elevate endogenous ceramide, thereby inducing apoptosis in tumor cells.

Apoptosis has two main signal transduction pathways: caspase dependent and mitochondria dependent. The caspase-dependent pathway begins to activate some death receptors and caspase-8, which is an initiator caspase. Once activated, the initiator caspase cleaves to and activates executioner caspases (caspase-3, -6, and -7), ultimately inducing cell death. On the other hand, the mitochondria-dependent pathway is considered to comprise at least three subsidiary death pathways. First, the release of cytochrome c from the mitochondrial intermembrane space initiates the classic caspase pathway, resulting in apoptotic cell death. This pathway partially overlaps the caspase-dependent and mitochondria-dependent pathways. Second, reactive oxygen species mediates necrosis-like cell death. Third, AIF, which is released from the mitochondrial intermembrane space, translocates into the cytoplasm and nucleus, resulting in apoptotic cell death. Many anticancer drugs induce cancer cell death by primarily using these pathways. Ceramide, however, reportedly induces apoptosis through both pathways mentioned earlier in cells such as those of acute myeloid leukemia. On the other hand, many human glioma cells demonstrate a resistance to ceramide, although a few researchers have reported that some cells resistant to radiation appears to be susceptible to ceramide. In this study we investigated the mechanisms of resistance to ceramide in human glioma cells and considered ways to overcome this resistance.

Materials and Methods

Cells Used in the Experiments

Human glioma cells (SKMG1, U251SP, U87MG, T98G, SKAO2, and U251MG) were maintained in Eagle medium containing 10% fetal bovine serum, 5 mM L-glutamine, and antibiotic agents (100
µg/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified atmosphere (95% air/5% CO₂).

Materials Used in the Study

The C2-ceramide was obtained from Upstate Biotechnology (Lake Placid, NY). For the immunochemical studies we used mouse mAb to AIF (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Alexa Fluor-488–labeled rabbit anti–mouse immunoglobulin G antibody (Molecular Probes, Inc., Eugene, OR), Mitotracker Red CMXRos (Molecular Probes, Inc.), and TO-PRO-3 iodide (Molecular Probes, Inc.).

Cell Viability

To evaluate the cytotoxicity of C2-ceramide, four kinds of human glioma cells, each containing 5000 cells in 100 µl of culture medium, were seeded into 96-well plates (BD Biosciences, San Jose, CA) and then incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 24 hours. After C2-ceramide treatment at a final concentration of 25 µM in 100 µl of serum-free medium, the viability of the cells was determined by performing a WST-1 assay (Roche Diagnostics, Indianapolis, IN) at a definite time. Ten microliters of WST-1 solution was added to each well. After incubation for 1 hour at 37°C, the absorbance difference between 450 and 655 nm was measured using a microplate reader (Benchmark Microplate Reader; Bio-Rad Laboratories, Hercules, CA) and the percentage of viability was calculated as follows: (experiment − blank)/(control − blank) × 100%, where experiment is the sample’s difference in absorbance between 450 and 655 nm, blank is a medium containing WST-1 solution without cells, and control is the signal from wells containing cells and 0.1% ethanol, but no serum medium.

Morphological Changes

Before C2-ceramide treatment, four kinds of human glioma cells were seeded into a glass-bottom culture dish (Mat Tec, Ashland, MA) and later incubated at 37°C in a humidified atmosphere of 95% air/5% CO₂ for 24 hours. After 24 hours of exposure to C2-ceramide (25 µM) or 0.1% ethanol, the glioma cells were observed with the aid of an inverted microscope equipped with a ×10 DIC objective lens and a ×1.6 insertion lens (Axiovert 135; Carl Zeiss, Göttingen, Germany). The microscopic images were sent to an image processor and controller via a CCD camera. Digitalized imaging data were captured with the aid of a computer imaging system (K5400 [version 3.00]; Carl Zeiss Vision GmbH, Hallbergmoos, Germany).

Assay of Caspase-3 and Caspase-8 Activities

A Caspase-3 and -8 Colorimetric Protease Assay Kit (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) was used to measure caspase-3 and -8 activities at various points after treatment. Cells treated with C2-ceramide were collected and lysed using cell lysis buffer, after which the supernatants were collected and measured using a DC Protein Assay kit (Bio-Rad Laboratories). Equal amounts of protein (50 µg) were diluted in 50 µl of cell-dilution buffer. Added to each sample was DEVD-pNa (caspase-3) or IETD-pNa (caspase-8) (final concentration of the substrate 200 µM). The samples were incubated at 37°C for 1 hour. After incubation, the optical density at 400 nm was measured using a microplate reader (Bio-Rad Laboratories).

Changes in the Mitochondrial Membrane Potential

To assess changes in the mitochondrial membrane potential, we used a tracking system (MitoTracker Orange CMTMRos; Molecular Probes, Inc.). Human glioma cells were seeded into a glass-bottomed culture dish and incubated at 37°C under humidified conditions for 24 hours. After changing to a dye-containing medium (Orange CMTMRos, 200 nM), the cells were incubated for 15 minutes at 37°C. After washing the labeled cells, minimal essential medium containing 25 µM C2-ceramide or 0.1% ethanol (control) was added to the dishes. Cells were observed using fluorescence microscopy and captured by the CCD camera after incubation. In each image, the cell bodies were circled, identifying them as regions of interest from which cellular fluorescence intensity could then be measured using imaging software (NIH Image [version 1.63]; National Institutes of Health, Bethesda, MD). Data were collected as background (cell-free regions)–subtracted fluorescence intensity.

Translocation of AIF

To confirm the localization of AIF protein, tumor cells (SKMG1 and U251SP) were grown on poly-L-lysine–coated glass coverslips (ASAHI Techno Glass Corp., Tokyo, Japan) in 24-well flat-bottom tissue culture plates (BD Biosciences) for 24 hours, after which the cells were exposed to C2-ceramide (25 µM) for an additional 24 hours. At the end of the incubation period, the cells were centrifuged on glass coverslips and stained for mitochondria with 50 nM Mitotracker Red CMXRos (Molecular Probes, Inc.) for 30 minutes at 37°C. The cells were then fixed for 15 minutes in 4% paraformaldehyde, washed in PBS, and permeabilized with 0.1% sodium dodecyl sulfate. The RNAs were digested by the addition of RNase (final concentration 0.5 mg/ml) to a blocking solution (PBS with 1% bovine serum albumin) for 30 minutes at room temperature. The cells were immersed in the blocking solution (30 minutes at room temperature) and incubated in AIF antibody solution (concentration 1:200) (2 hours at room temperature). They were then washed with three changes of PBS and incubated in Alexa Fluor 488–labeled secondary antibody solution (concentration 1:300) containing TO-PRO-3 iodide (final 1 µM) for 1 hour at room temperature. The negative control consisted of slides to which only the secondary antibody containing TO-PRO-3 iodide was added. Finally, coverslips were washed in PBS, mounted on glass slides over a drop of Perma Fluor (Immuno, Pittsburgh, PA), and stored at 4°C in the dark until observation. The slides were examined under a Nikon Eclipse E600 microscope with a Plan Apo 60×/1.4 oil immersion lens (Nikon Corp., Tokyo, Japan). The system was equipped with a laser confocal device (Radiance 2000; Bio-Rad Laboratories), composed of an Argon ion laser (488 nm excitation), a green HeNe laser (543 nm excitation), a red laser diode (638 nm excitation), and filters (HQ530/60, HQ660/50, and HQ660LP). Image processing was performed using LaserSharp 2000 computer software (Bio-Rad Laboratories).

Western Blot Analysis

Treated cells were collected by scraping in PBS and were pelleted before lysing in a buffer containing the protease inhibitors phenylmethylsulfonyl fluoride (100 µg/ml), aprotinin (1 µg/ml), and leupeptin (1 µg/ml). Lysate protein concentrations were determined using a Bio-Rad DC Protein Assay kit. Protein samples were denatured in gel-loading buffer (New England Biolabs, Inc., Beverly, MA) at 99°C for 5 minutes. Equal amounts of protein (65 µg) were applied to each well and electrophoresed on 7.5% polyacrylamide gel or 14 to 16% polyacrylamide gradient gel (both from Daiichi Pure Chemicals, Ltd., Tokyo, Japan). Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Billerica, MA); blocked with 10% lowfat milk; and incubated with primary antibody against caspase-7 mAb (clone 4G2; Pharmingen, San Diego, CA), caspase-3 polyclonal antibody (Phar-mingen), FARP polyclonal antibody (Cell Signaling Technology, Inc., Beverly, MA), and XIAP mAb (clone 48; Pharmingen), after which the proteins were washed and blotted with species-specific secondary antibodies (mouse and rabbit) (Santa Cruz Biotechnology). The blotted proteins were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

Determination of DNA Fragmentation

We monitored the C2-ceramide–induced intranucleosomal DNA fragmentation. Cultured human glioma cells (10⁶) were treated with 0.1% ethanol or 25 µM C2-ceramide for 12, 24, and 48 hours. To evaluate intranucleosomal DNA fragmentation, we used the Apoptosis Ladder Detection Kit (Wako Pure Chemical Industries, Osaka, Japan). Glioma cells were incubated with 25 µM C2-ceramide or 0.1% ethanol for 12 to 48 hours. Genomic DNA was extracted according to the manufacturer’s protocol. The extracted DNA was
Small Interfering RNA Transfection

Tumor cells were transfected with siRNA for XIAP messenger RNA in accordance with the following protocol. The siRNA sequences targeting XIAP (Gene Bank No. U45880) corresponded to coding regions 111 to 131 relative to the first nucleotide of the start codon. The nucleotides included sense 5'–GUCCGUAGUCCGU–3' and antisense 5'–GCCUGAAACAGGACU–3'. The RNAs were chemically synthesized by JBioS, Inc. (Saitama, Japan). For the annealing of dsRNA, 100 μM sense and antisense RNAs were incubated in annealing buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES-KOH at pH 7.4) for 1 minute at 90°C, 1 minute at 70°C, and followed by 1 hour at 37°C. The day before transfection, 10^5 trypsinized cells were plated to each well of six-well Falcon plates (BD Biosciences). After 24 hours of plating, dsRNA was mixed with Oligofectamine reagent (Invitrogen, Carlsbad, CA) in OptiMEM medium (Gibco-BRL, Gaithersburg, MD). Cultured cells were washed with medium without serum and added to the dsRNA–Oligofectamine mixture (final concentration 50 nM dsRNA). Up to 10% fetal bovine serum was added to the medium 4 hours later. After the cells were incubated at 37°C under humidified conditions for 48 hours, they were treated with 25 μM C2-ceramide and incubated for various periods (6–36 hours). The cells were then lysed in lysis buffer and the lysates were subjected to Western blot analysis for expressions of caspase-7, caspase-3, PARP, and XIAP.

Results

Antitumor Activity of C2-Ceramide in Human Glioma Cells

To screen human glioma cells for their susceptibility to C2-ceramide, SKMG1, U87MG, U251SP, and T98G cells were exposed to C2-ceramide for various periods. Both SKMG1 and U87MG cells demonstrated a high susceptibility to C2-ceramide, resulting in cell death (Fig. 1). By 18 hours, cell viability had decreased 40% from that of control. In contrast, U251SP and T98G cells demonstrated a high resistance to C2-ceramide (Fig. 1).
FIG. 2. Mitochondrial membrane potential of human glioma cells exposed to C2-ceramide. Both SKMG1 and U251SP cells were labeled with MitoTracker Orange CMTMRos (200 nM) for 15 minutes following exposure to C2-ceramide (25 μM, black bars) or ethanol (0.1%, open bars). Cellular fluorescence images were captured by a CCD camera after 1-, 9-, and 16-hour incubations. Cellular fluorescence intensity was measured with the aid of NIH Image software. Data represent the means ± standard deviations of 200 cells. Percentages of intensity are calculated as described in Materials and Methods. * p < 0.01.

FIG. 3. Laser confocal images of immunocytochemical findings. The AIF was stained with anti-AIF mAb and Alexa Fluor-488 (green signals)–labeled secondary antibody. Nuclear DNA was stained with TO-PRO-3 (blue signals). Confocal laser microscopy reveals that control cells show a perinuclear localization of AIF (upper). The C2-ceramide induced the appearance of AIF in the cytosol and nucleus, as evidenced by the fusion image (light blue signals; arrow) in SKMG1 cells (lower left). Sections through the center of the cells are shown.
Morphological Changes in Human Glioma Cells Exposed to C2-Ceramide

Morphological changes in human glioma cells exposed to C2-ceramide were observed for 24 hours by performing DIC microscopy. Compared with the 0.1% ethanol–treated cells, those cells exposed to C2-ceramide underwent morphological changes, for example, membrane blebbing, ballooning, and the appearance of apoptotic bodies in SKMG1 (Fig. 1A-2) and U87MG (Fig. 1B-2) cells. In other human glioma cells (U251SP and T98G cells), no morphological changes occurred with or without C2-ceramide exposure (Fig. 1C-2 and D-2).

Caspase-3 and Caspase-8 Activities During Exposure to C2-Ceramide

To determine whether C2-ceramide induction of cell death depended on its caspase pathway, we measured the activities of caspase-3 and -8 in four groups of glioma cells exposed to C2-ceramide. Treatment of those cells with 25 μM C2-ceramide resulted in no significant increases in caspase-3 and -8 activities compared with control cells (data not shown). Even when cells from the C2-ceramide–sensitive cell lines SKMG1 and U87MG died, neither caspase-3 nor caspase-8 was activated (data not shown).

Mitochondrial Membrane Potential of Human Glioma Cells Exposed to C2-Ceramide

To evaluate whether mitochondrial conditions differed between C2-ceramide–sensitive (SKMG1) and –resistant (U251SP) cells, the mitochondrial membrane potential was assessed using MitoTracker Orange CMTMRos staining. Treatment of cells with 25 μM C2-ceramide caused a significant increase in mitochondrial fluorescence after 9 hours in the sensitive SKMG1 cells (Fig. 2); however, the resistant U251SP cells displayed the same intensity as control cells at various incubation times (Fig. 2). These data indicated that the mitochondrial membrane potential in SKMG1 cells was reduced by exposure to C2-ceramide.

Translocation of AIF Into the Nucleus

Confocal laser microscopy revealed that AIF translocated into the nucleus after 24 hours of incubation in SKMG1 cells exposed to C2-ceramide (Fig. 3). Light blue regions demonstrated green (Alexa Fluor-488) overlapping the blue (TO-PRO-3), indicating that AIF had translocated into the nucleus (Fig. 3 lower left). In contrast, control SKMG1 cells showed a perinuclear localization of AIF (Fig. 3 upper left). The C2-ceramide–resistant U251SP cells showed no translocation of AIF into the nucleus (Fig. 3 upper and lower right).

Fragmentation of DNA in Human Glioma Cells Exposed to C2-Ceramide

Cell death induced by C2-ceramide was morphologically indicative of the progression of apoptosis. In fact, internucleosomal DNA fragmentation was observed in C2-ceramide–sensitive SKMG1 cells (Fig. 4 left). Nevertheless, C2-ceramide induced no DNA fragmentation whatsoever in resistant U251SP cells (Fig. 4 right).

Caspase-7 and PARP in Human Glioma Cells Exposed to C2-Ceramide

Western blot analysis confirmed the expression of 35-kD procaspase-7, 116-kD full-length PARP (Fig. 5), and 33-kD...
procaspase-3 (data not shown) under control conditions in both SKMG1 and U251SP cells. Following treatment with C2-ceramide, there was a time-dependent cleavage of caspase-7 and a downregulation of full-length PARP in C2-ceramide–sensitive SKMG1 cells (Fig. 5 left); no such cleavage of caspase-7 or downregulation of PARP was expressed in C2-ceramide–resistant U251SP cells (Fig. 5 right).

Expression of XIAP in Human Glioma Cells

Western blot analysis was performed to verify XIAP expression in human glioma cells (SKMG1, U87MG, U251SP, T98G, U251-MG, and SKAO2), mouse melanoma cells (B16F1), and human T-cell leukemia cells (Jurkat). Even if the applied protein volume was the same in each line, the XIAP protein levels were much higher in human glioma cell lines than in the other two types of cells (Fig. 6A).

Effect of XIAP Inhibition on C2-Ceramide–Resistant U251SP Cells

To determine whether the inhibition of XIAP was effective in inducing cell death in C2-ceramide–resistant cell
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Caspase-7

PARP

Fig. 7. Activation of caspase-7 and PARP after exposure of XIAP-inhibited U251SP cells to C2-ceramide. The U251SP cells, which were untreated (siRNA−) or treated (siRNA+) with siRNA, were exposed to C2-ceramide (25 μM) for 6 to 36 hours. Immunoblot analyses of caspase-7 and PARP were performed as described in Materials and Methods. This experiment was repeated three times and the same results were obtained each time. Representative data are shown in this figure.

Discussion

In the present study, in contrast to hematopoietic cells, C2-ceramide could not induce apoptosis in the majority of human glioma cells except for SKMG1. The detailed mechanisms of resistance to ceramide in human glioma cells remain poorly understood.

In C2-ceramide–sensitive human glioma cells (SKMG1 cells), caspase-7 was activated, but caspase-8 and -3 were not. In those cells, the loss of the mitochondrial membrane potential and AIF translocation into the nucleus were also detected, resulting in a high-molecular-mass DNA fragmentation and marginal chromatin condensation. The same results were observed by Susin, et al.32 The role of caspase-7 activation still remains unknown in C2-ceramide–sensitive human glioma cells, however, because caspase-3, which is located downstream from caspase-7, was not activated at all. Some investigators have reported on chemotherapeutic drug-induced apoptosis of human malignant glioma cells involving the death receptor-independent activation of caspases, except for caspase-8 and -3, and, in almost all of the cases, noted that mitochondrial cytochrome

Electrophoretic patterns of oligonucleosomal DNA laddering were detected in U251SP cells treated with both siRNA and C2-ceramide, indicating that C2-ceramide could induce apoptotic cell death in C2-ceramide–resistant human glioma cells (Fig. 8).

Fig. 8. Induction of DNA fragmentation by inhibiting XIAP. An analysis of DNA fragmentation of U251SP cells by agarose gel (1.5%) electrophoresis 24 hours after treatment with C2-ceramide (C2) or C2-ceramide and siRNA targeting of the XIAP gene (C2 + siRNA) is shown. M = molecular weight marker (100-bp ladder marker). This experiment was repeated three times and the same results were obtained each time. Representative data are shown in this figure. SYBR Green I.
c release was required. On the other hand, in C2-ceramide–resistant human glioma cells (U251SP and T98G cells), C2-ceramide failed to activate caspase-8, -3, or -7. These results indicate that usual apoptotic pathways, which were induced by some death receptors and caspases, played no role in C2-ceramide–resistant human glioma cells.

We also investigated the expression in human glioma cells of IAPs, which are a counterpart to caspases. The IAP family is defined by a novel conserved motif termed the “baculoviral IAP repeat.” In humans, six IAP-like proteins have been identified and designated XIAP, HIAP-1, HIAP-2, neuronal apoptosis inhibitory protein, survivin, and apoptosis-2. The most potent caspase inhibitor, XIAP, directly binds and inhibits caspase-3, -7, and -9. Recent studies have demonstrated that the downregulation of XIAP by using an antisense XIAP-expressing adenovirus effectively induces apoptosis in chemoresistant ovarian cancer cells. The proteins XIAP, HIAP-1, and HIAP-2 are widely expressed by glioma cell lines, and it has been also reported that IAPs are often upregulated in brain tumors. These proteins may play a role in the striking resistance of gliomas to the induction of apoptosis by radiotherapy and chemotherapy. Such findings indicate that human glioma cells resistant to C2-ceramide may be able to induce apoptosis via XIAP downregulation. As another possibility, there are some reports that the C2-ceramide–mediated apoptosis of gliomas was inhibited by Bcl-2.

Next we investigated the efficacy of IAP inhibition in human glioma cells to overcome the resistance to apoptosis. First, we tried to knock down XIAP in C2-ceramide–resistant human glioma cells by using a newly established approach involving siRNA, reported by Elbashir, et al. The transfection of siRNA targeting of the XIX gene remarkably reduced the production of XIAP protein in human glioma cells. The C2-ceramide–resistant U251SP cells initially inhibited XIAP production by siRNA and then exposed to C2-ceramide, displayed apoptotic events, that is, activation of caspase-7, cleavage of PARP, and DNA fragmentation (Figs. 7 and 8).

In conclusion, inhibition by endogenous caspase inhibitors, such as XIAP, can render C2-ceramide–resistant human glioma cells sensitive to C2-ceramide through the activation of caspase-dependent pathways.

References

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Address reprint requests to: Jun Yoshida, M.D., Ph.D., Department of Neurosurgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466–8550, Japan. email: jyoshida@med.nagoya-u.ac.jp.