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Ordering of ceramide formation, caspase activation, and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells

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Abstract

Etoposide (VP-16) a topoisomerase II inhibitor induces apoptosis of tumor cells. The present study was designed to elucidate the mechanisms of etoposide-induced apoptosis in C6 glioma cells. Etoposide induced increased formation of ceramide from sphingomyelin and release of mitochondrial cytochrome *c* followed by activation of caspase-9 and caspase-3, but not caspase-1. In addition, exposure of cells to etoposide resulted in decreased expression of Bcl-2 with reciprocal increase in Bax protein. z-VAD·FMK, a broad spectrum caspase inhibitor, failed to suppress the etoposide-induced ceramide formation and change of the Bax/Bcl-2 ratio, although it did inhibit etoposide-induced death of C6 cells. Reduced glutathione or *N*-acetylcysteine, which could reduce ceramide formation by inhibiting sphingomyelinase activity, prevented C6 cells from etoposide-induced apoptosis through blockage of caspase-3 activation and change of the Bax/Bcl-2 ratio. In contrast, the increase in ceramide level by an inhibitor of ceramide glucosyltransferase-1, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol caused elevation of the Bax/Bcl-2 ratio and potentiation of caspase-3 activation, thereby resulting in enhancement of etoposide-induced apoptosis. Furthermore, cell-permeable exogenous ceramides (C_2- and C_6-ceramide) induced downregulation of Bcl-2, leading to an increase in the Bax/Bcl-2 ratio and subsequent activation of caspases-9 and -3. Taken together, these results suggest that ceramide may function as a mediator of etoposide-induced apoptosis of C6 glioma cells, which induces increase in the Bax/Bcl-2 ratio followed by release of cytochrome *c* leading to caspases-9 and -3 activation. *Cell Death and Differentiation* (2000) **7,** 761–772.

Keywords: apoptosis; Bcl-2 family; caspase; ceramide; glioma

Abbreviations: SMase, sphingomyelinase; SM, sphingomyelin; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NAC, *N*-acetylcysteine; GSH, reduced glutathione; PDMP, D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; GlcT-1, UDP-glucose: ceramide glycosyltransferase-1; HPTLC, high performance thin-layer chromatography; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; AMC, 7-amino-4-methylcoumarin; RT–PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; FB1, fumonisin B1; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species

Introduction

Recent studies have shown that a variety of DNA-damaging agents initiate the pathways leading to apoptosis. Etoposide, an inhibitor of DNA topoisomerase II is one of the DNA-damaging agents and has a potent anticancer action. Thus, etoposide has been used clinically both as a single agent and a constituent of combination chemotherapy regimens for the treatment of malignant brain tumors. Several reports using hematopoietic cells including thymocytes,[1] lymphocytes,[2] and leukemic cells[3,4] have demonstrated that etoposide induces apoptotic cell death. However, the exact molecular mechanism leading to apoptotic cell death by etoposide remains to be resolved. Furthermore, it is not clear whether the results obtained from hematopoietic cells are applicable to non-hematopoietic cells including glioma cells.

Recently, several components involving apoptotic cell death have been extensively studied including ceramide, caspases and the Bcl-2 family. Ceramide is a product of sphingomyelinase (SMase)-catalyzed hydrolysis of sphingomyelin (SM). Formation of ceramide has been regarded as a key pathway involved in apoptosis as well as differentiation.[5,6] The ability of ceramide to induce apoptosis has been demonstrated in many cell types[7–9] including cells of central nervous system such as cultured neurons,[10] oligodendrocytes,[11] and astrocytes,[12] although several recent biochemical analyses have questioned the role of ceramide during apoptosis induced by Fas or tumor necrosis factor (TNF)-α.[13–17] We have previously reported that membrane permeable ceramide analogs, C_2- and C_6-ceramide induce apoptosis of C6 glioma cells.[18] Furthermore, a close relationship between ceramide-mediated signaling and apoptosis of C6 cells has recently been reported.[19] These findings imply that ceramide may function as an important modulator of glial apoptosis.

Caspases are a family of cysteine proteases, which are homologous to the product of the *Caenorhabditis elegans*

cell death gene ced3.[20] More than 10 caspases have been identified in mammals and are classified into three subfamilies, caspase-1-, caspase-2-, and caspase-3 (CPP32/Yama/apopain)-like proteases, based on their structures.[21] Recent studies suggest the presence of two major caspase cascades. One is initiated by activation of cell-surface death receptors, such as Fas and TNF-α receptor, leading to caspase-8 activation, which in turn cleaves and activates the downstream caspases such as caspases-3, -6, and -7.[22–24] The other is triggered by cytochrome *c* released from mitochondria, which promotes formation of a multi-subunit complex with Apaf-1 and procaspase-9, leading to caspase-9 activation.[25] Active caspase-9 subsequently activates caspases-3, -6, and -7.[26,27] The latter caspase cascade is proposed to be responsible for induction of chemical- or irradiation-induced apoptosis in many types of cells.[28–30] Apoptosis is also regulated by the Bcl-2 family proteins, including anti-apoptotic proteins such as Bcl-2 and Bcl-X_L, and pro-apoptotic proteins such as Bax and Bak.[31–33] Bcl-2 has been known to modulate susceptibility to etoposide-induced apoptosis of mouse bone marrow cells and B cells.[34,35]

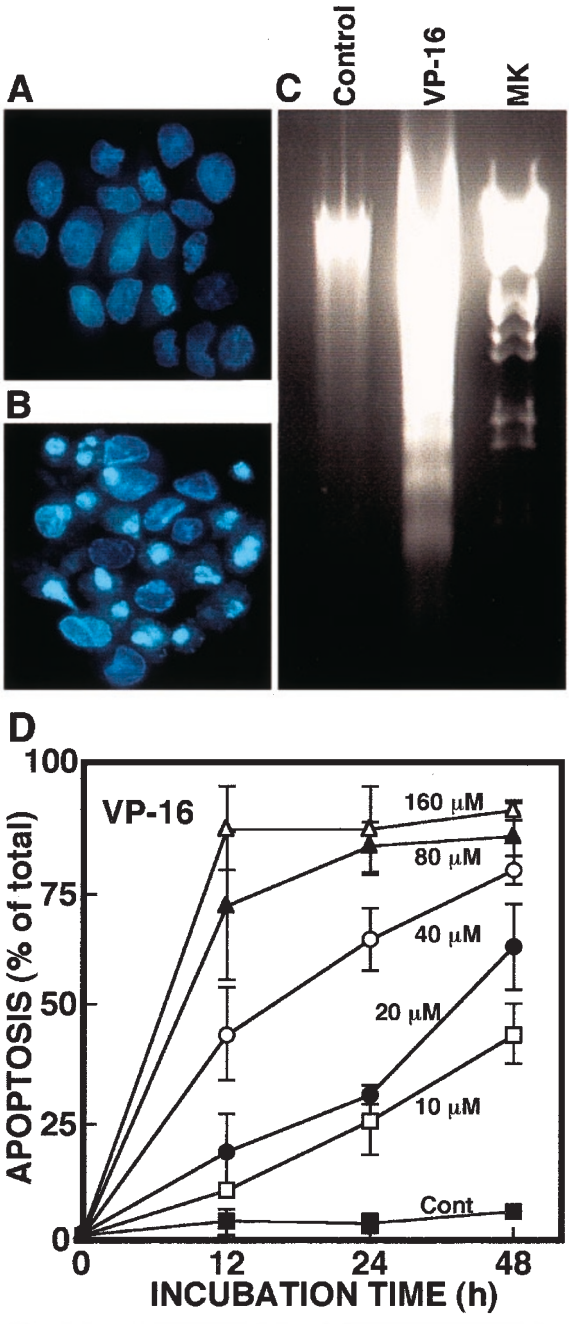

Figure 1 Etoposide-induced apoptotic cell death in C6 cells. (**A**) Untreated cells. (**B**) Cells treated with 40 μM etoposide (VP-16) for 24 h. Cells were stained with Hoechst 33258 (10 μM) for 10 min and photographed under a fluorescent microscope. Photographs are representatives from at least 10 different cultures. (**C**) Agarose gel electrophoresis of oligonucleosomal DNA fragments (DNA laddering). After 24 h exposure of C6 cells to 40 μM etoposide (VP-16), DNA extracted from the cells was subjected to conventional agarose gel electrophoresis. MK: 1 Kb ladder molecular weight marker. (**D**) C6 cells were treated with different concentrations of etoposide (VP-16) for indicated periods. The cells with fragmented and condensed nuclei were counted in over 1000 cells under a fluorescent microscope. Data are means $\pm$ S.D. from three independent experiments, each performed in triplicate

In the present study, we have examined the molecular events leading to apoptosis in etoposide-treated C6 glioma cells. Etoposide induced the changes of expression of Bcl-2 and Bax proteins, release of mitochondrial cytochrome *c* and activation of caspases-9 and -3. In addition, ceramide formation by SM hydrolysis was observed during the apoptotic process. To date, however, the interrelationship among expression of Bcl-2 family proteins, caspase activation, and ceramide formation has not yet been understood. Therefore, we have further attempted to examine their sequential relation in the etoposide-induced glial apoptosis. The date obtained in the present study suggest that ceramide induces change of the Bax/Bcl-2 ratio, which regulates cytochrome *c* release from mitochondria, leading to activation of caspase-9/-3 cascade.

Results

Etoposide induced apoptotic cell death in C6 cells

We first examined the cytotoxic effects of etoposide (VP-16) on C6 glioma cells under phase contrast microscopy. Marked morphological changes were observed at 12 h after the treatment with 40 μM etoposide. Cellular processes were retracted and the rounded cells were detached from culture dishes. The nuclear staining with Hoechst 33258 revealed typical apoptotic changes, condensation and fragmentation of nuclei (Figure 1A,B). Further support to indicate apoptotic change was provided by the characteristic pattern (laddering) of DNA fragmentation (Figure 1C). For quantitative analysis, the cells with apoptotic morphological features were counted among over 1000 cells under fluorescent microscopy. Treatment of C6 cells with etoposide induced a concentration- and time-dependent increase in apoptotic cells (Figure 1D).

Activation of SMase and formation of ceramide during etoposide-induced cell death

Recently, neutral and acid SMases have been demonstrated to involve the formation of ceramide in response to apoptotic

inducers including chemotherapeutic agents.[6,36] Therefore, changes of both neutral and acid SMase activities were measured in cells treated with 40 μM etoposide (Figure 2). Etoposide gave rise to a sharp increase in neutral SMase activity at 1 h after treatment and its activity increased thereafter in a time-dependent manner. On the other hand, significant changes were not observed in acid SMase activity, although its activity was higher than that of neutral one during time course examined.

In order to further assess the activation of neutral SMase, the levels of intracellular ceramide during etoposide-treatment were measured by the enzymic analysis with *E. coli* diacylglycerol kinase and also by the metabolic labeling of cells with [^{14}C]serine. A marked increase in ceramide content was observed as early as 1 h after the etoposide treatment by the diacylglycerol kinase assay (Figure 3A). The maximal level was observed at 24 h (threefold increase over the control level). Similar profile of ceramide formation was observed in cells labeled with [^{14}C]serine (Figure 3B). Concurrently, a sharp decrease in [^{14}C]SM was observed as early as 3 h after etoposide-treatment (Figure 3C) and its level was declined to approximately 30% of the control level at 24 h.

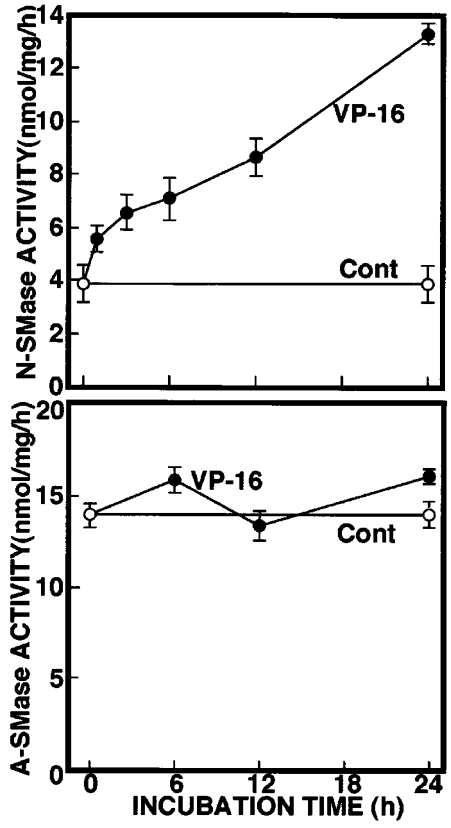

Figure 2 Changes in neutral and acid SMase activities in C6 cells exposed to etoposide. C6 cells were treated with 40 μM etoposide (VP-16) for indicated periods. The activities of neutral SMase (N-SMase) and acid SMase (A-SMase) were determined using a mixed micelle assay system with [*methyl*-^{14}C]SM at pH 7.5 and 5.5, respectively, as described in Materials and Methods. Data are means $\pm$ S.D. from two independent experiments, each performed in triplicate

Moreover, to confirm the pathway of ceramide formation, we investigated the effect of fumonisin B1 (FB1), an inhibitor of sphinganine *N*-acetyltransferase, which is known to inhibit *de novo* synthesis of ceramide.[37,38] However, 100 μM FB1 had no effect on etoposide-

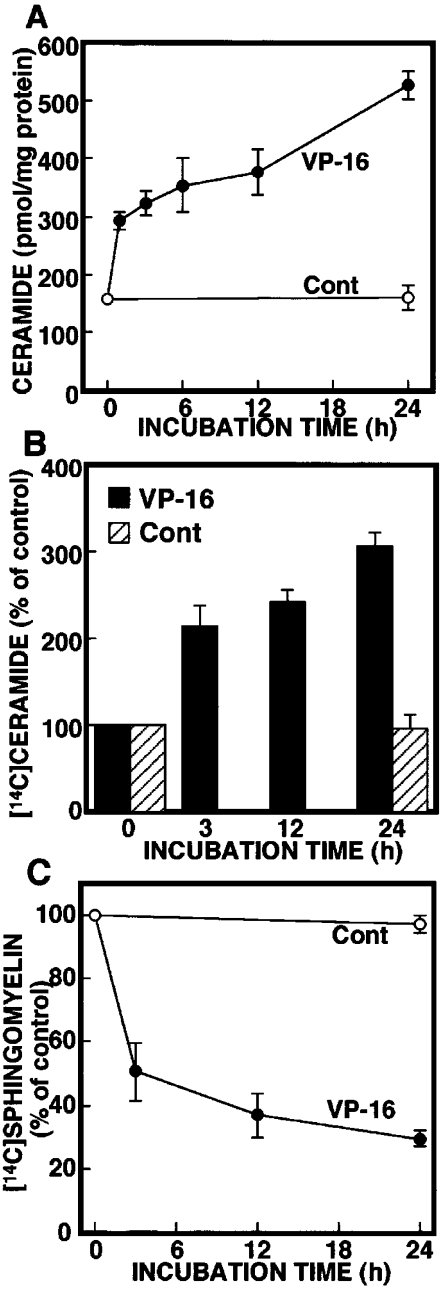

Figure 3 Etoposide-induced ceramide formation in C6 cells. C6 cells were treated with 40 μM etoposide (VP-16) for indicated periods. (**A**) Ceramide content was measured by the *E. coli* diacylglycerol kinase assay as described in Materials and Methods. Data are means $\pm$ S.D. from two independent experiments, each performed in triplicate. (**B,C**) Changes in [^{14}C]ceramide (B) and [^{14}C]SM (C) in [^{14}C]serine-labeled cells were measured as described in Materials and Methods. The radioactivity in [^{14}C]ceramide (422 $\pm$ 19 d.p.m.) and [^{14}C]SM (7,670 $\pm$ 26 d.p.m.) in unstimulated control (Time 0) was designated as 100%. Data are means $\pm$ S.D. from two independent experiments

induced formation of ceramide and increase in the number of apoptotic cells (data not shown). These results indicate that during etoposide-induced apoptosis, ceramide is produced from SM hydrolysis, but not from *de novo* synthesis.

Caspase activation and cytochrome *c* release from mitochondria by etoposide

The caspase family has been suggested to play a pivotal role in apoptosis.[20] To identify the apoptotic mechanism of etoposide-induced cell death, caspase-1(-like) and caspase-3(-like) activities were measured spectrophotometrically following the etoposide treatment. Etoposide failed to activate caspase-1(-like) protease measured as DMQD cleavage during the time course examined (Figure 4A). In contrast, the DEVD cleaving activity began to increase from 6 h and reached a peak at 12 h after etoposide treatment (Figure 4B), indicating activation of caspase-3(-like) protease. The maximal activity was maintained up to 24 h and then gradually decreased.

We further examined the processing of caspases by immunoblot analysis (Figure 4C). Immunoreactive 45-kDa procaspase-1 (ICE) remained unchanged during the first 6 h and was slightly decreased thereafter, probably due to nonspecific proteolysis, since DMQD cleaving activity was not upregulated as shown above. In contrast, the levels of 32-kDa procaspase-3 (CPP32) began to be degraded as early as 6 h after the etoposide treatment, which were well correlated with the changes of DEVD cleaving activity measured spectrophotometrically. Processing of caspase-3 was also confirmed by the time-dependent degradation of poly (ADP-ribose) polymerase (PARP), a well-known substrate of caspase-3 (data not shown). In addition, immunoreactive proform of caspase-9, which is known as an upstream activator of caspase-3, was processed at 6 h after etoposide treatment to yield active fragments. Activation of caspase-9 is shown to be triggered by the release of cytochrome *c* from mitochondria to cytosol.[25] Immunoblot analysis revealed that cytochrome *c* accumulated in cytosol at 3 h after exposure to etoposide (Figure 4D). Therefore, cytochrome *c* release from mitochondria preceded the processing of procaspases-9 and -3.

Changes in mRNA and protein levels of Bcl-2 and Bax during etoposide-treatment

Apoptotic cell death is also known to be regulated by Bcl-2 family proteins.[31,32] The fate of cells is thought to be determined by the balance between pro-apoptotic and anti-apoptotic members.[39–41] To examine involvement of Bcl-2 and Bax in etoposide-induced apoptosis, changes in their mRNA and protein levels were examined by RT–PCR and Western blotting, respectively. RT–PCR analysis revealed that mRNA level of *bax* time-dependently increased with a reciprocal decrease of *bcl-2* mRNA (Figure 5A). No significant changes were observed in the level of GAPDH mRNA (reference control) throughout the time course examined.

By Western blot analysis, the etoposide-treatment was found to cause downregulation of Bcl-2 and upregulation of Bax in a time-dependent manner (Figure 5B). The actin level, as reference control, indicated the equal amount of protein loaded in each lane. The densitometric analysis revealed that changes in the mRNA levels of *bcl-2* and *bax* were well correlated with those in Bcl-2 and Bax protein expression.

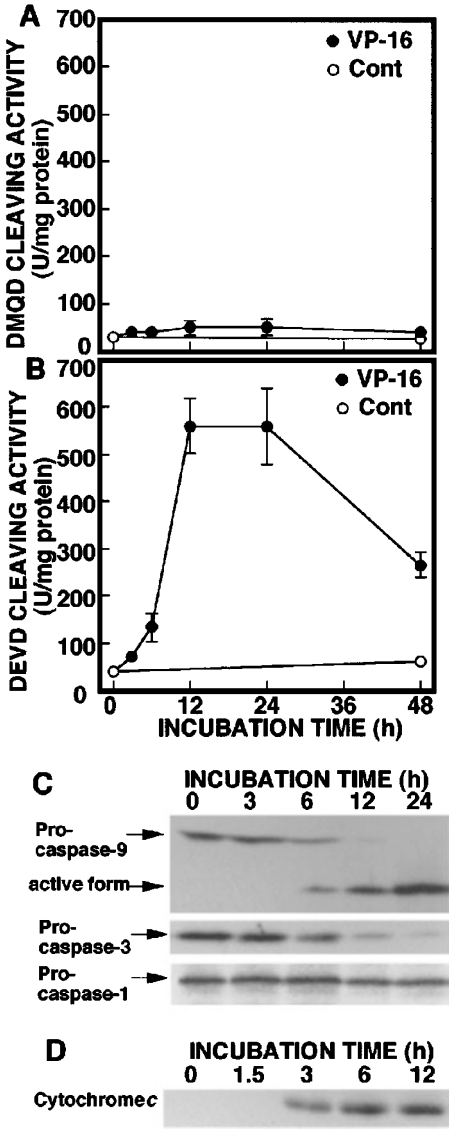

Figure 4 Activation of caspase proteases and release of mitochondrial cytochrome c by etoposide. (**A,B**) C6 cells were harvested after exposure to 40 μM etoposide (VP-16) for indicated periods. The cellular extracts (40 μg of protein) were incubated with 50 μM Ac-DMQD-MCA, a substrate for caspase-1(-like) protease (**A**) or 50 μM Ac-DEVD-MCA, for caspase-3(-like) protease (**B**) at 37°C for 1 h. Levels of released 7-amino-4-methylcoumarin were measured using a spectrofluorometer as described in Materials and Methods. Data are means $\pm$ S.D. from three independent experiments, each performed in triplicate. (**C,D**) Processing of caspases (**C**) and release of cytochrome *c* from mitochondria to cytosol (**D**) were analyzed by Western blot analysis as described in Materials and Methods. Results are representative of two separate experiments with compatible outcomes

Inhibition of etoposide-induced apoptosis and activation of caspase-3, but not ceramide formation by a caspase inhibitor

Ceramide accumulation, increase in Bax/Bcl-2 ratio and activation of caspase-3 were also observed in a human glioblastoma U87-MG cells exposed to etoposide (data not shown). Therefore, these events are involved in etoposide-induced apoptosis of glioma cells. To investigate their sequential relationship, the effects of z-VAD·FMK, a broad spectrum caspase inhibitor, was first examined for apoptosis and ceramide formation. The increase in number of apoptotic C6 cells induced by etoposide was concentration-dependently suppressed by z-VAD·FMK and was decreased to less than 20% of the total cells at 200 μM z-VAD·FMK (Figure 6A). Preincubation of C6 cells with z-VAD·FMK inhibited the etoposide-induced activation of caspase-3. In particular, in the presence of 200 μM z-VAD·FMK, the DEVD cleaving activity was reduced almost to the control level and cleavage of procaspase-3 was almost completely prevented (data not shown). These findings imply that the ability of the caspase inhibitor to suppress etoposide-induced apoptosis is due to inhibition of caspase-3 activation. However, the etoposide-induced formation of ceramide was not affected by z-VAD·FMK even at 200 μM (Figure 6B).

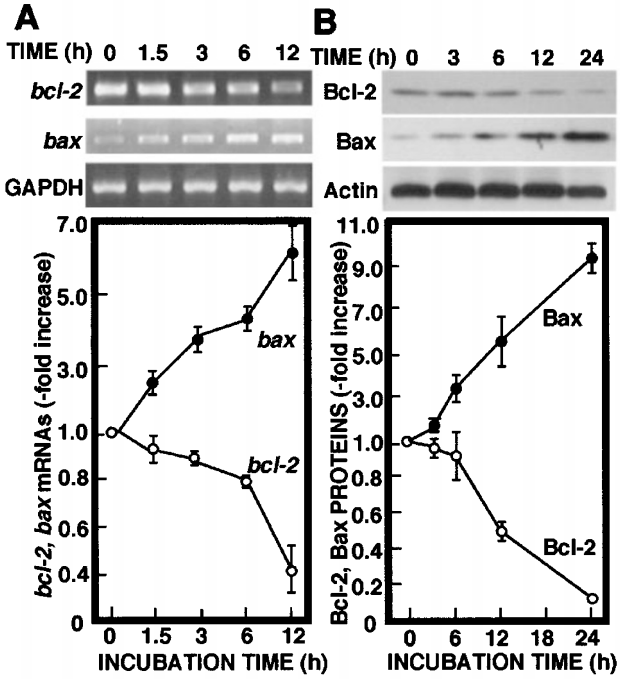

Figure 5 Changes in mRNA and protein levels of Bcl-2 and Bax in C6 cells exposed to etoposide. (**A**) After C6 cells were treated with 40 μM etoposide for indicated periods, total RNA was isolated with Isogen. Levels of mRNAs were analyzed by RT–PCR as described in Materials and Methods. (**B**) Cellular proteins were subjected to SDS–PAGE and immunoblotted with antibodies against Bcl-2, Bax, or actin. Visualization of the proteins was performed with ECL. Intensity of bands was quantified by a densitometer. Data represent means $\pm$ S.D. values from three different experiments, each performed in duplicate

Ceramide content affects etoposide-induced apoptosis and activation of caspase-3

To examine whether changes in ceramide content affect etoposide-induced apoptosis and caspase-3 activation, we used reduced glutathione (GSH) and *N*-acetylcystetine (NAC) which are known to inhibit SMase activation by various stimuli.[42–44] Preincubation of C6 cells with GSH or NAC reduced ceramide accumulation induced by etoposide (Figure 7A). Under the same condition, the etoposide-induced activation of DEVD cleaving activity was also inhibited (Figure 7B). Moreover, degradation of procaspase-3 was inhibited (data not shown). In a similar fashion, GSH or NAC concentration-dependently rescued cells from apoptosis and at 25 mM GSH or NAC, the percentage of apoptotic cells was reduced from 60 to about 20% (data not shown).

Recently, the involvement of UDP-glucose: ceramide glucosyltransferase-1 (GlcT-1) has been suggested in

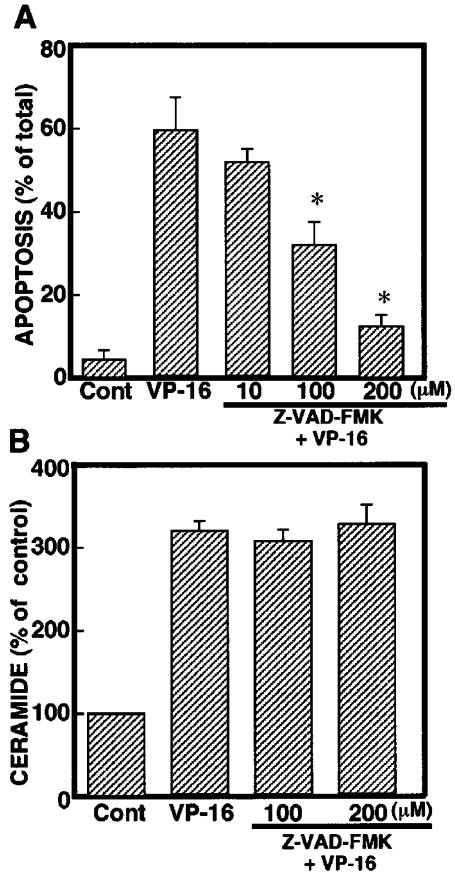

Figure 6 Effects of a caspase inhibitor in etoposide-induced apoptosis and formation of ceramide. C6 cells were preincubated with different doses of z-VAD·FMK for 1 h and then exposed to 40 μM etoposide (VP-16) for 24 h. (**A**) Effect of z-VAD·FMK on the percentage of the apoptotic cells. The cells with fragmented or condensed nuclei were counted in over 1000 cells under fluorescent microscopy. (**B**) Ceramide content in C6 cells treated with etoposide in combination with or without z-VAD·FMK. Ceramide content was measured by the *E. coli* diacylglycerol kinase assay as described in Materials and Methods. Data are means $\pm$ S.D. from two independent experiments, each performed in triplicate. *$P<0.01$ *versus* VP-16 alone: ANOVA with Scheffe's *post-hoc* test

regulation of the intracellular ceramide level, especially when it is produced in excess. D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a potent inhibitor of GlcT-1, gives rise to increase of the ceramide content in B16 melanoma cells.[45] We next tested the effects of PDMP in C6 glioma cells. As expected, preincubation of C6 cells with PDMP increased the etoposide-induced ceramide level (Figure 8A). The ceramide content in the cells preincubated with 100 μM PDMP increased to approximately 180% of that in the cells treated with etoposide alone. In the presence of 100 μM PDMP, the etoposide-induced DEVD cleaving activity was also elevated to 1.2-fold (Figure 8B). PDMP concentration-dependently potentiated apoptosis induced by etoposide (data not shown). These results collectively suggest that the ceramide level may regulate etoposide-induced apoptosis by affecting caspase-3 activity.

Regulation of Bax/Bcl-2 expression by ceramide content but not by caspase-3 activity

We further investigated the roles of ceramide and caspase-3 in the changes of the Bax/Bcl-2 ratio. The effect of z-VAD·FMK on Bcl-2 and Bax expression was first examined. However, 200 μM z-VAD·FMK, which most effectively inhibited etoposide-induced apoptosis, did not affect the Bax/Bcl-2 expression by etoposide (Figure 9A). These findings indicate that the changes in Bcl-2 and Bax expression do not occur downstream of caspase-3 activation. Furthermore, these results imply that the observed changes in Bcl-2 and Bax expression may not be the consequence of cell death.

We next examined the relationship between ceramide formation and Bax/Bcl-2 expression using GSH, NAC or PDMP. To be of interest, blockage of etoposide-induced

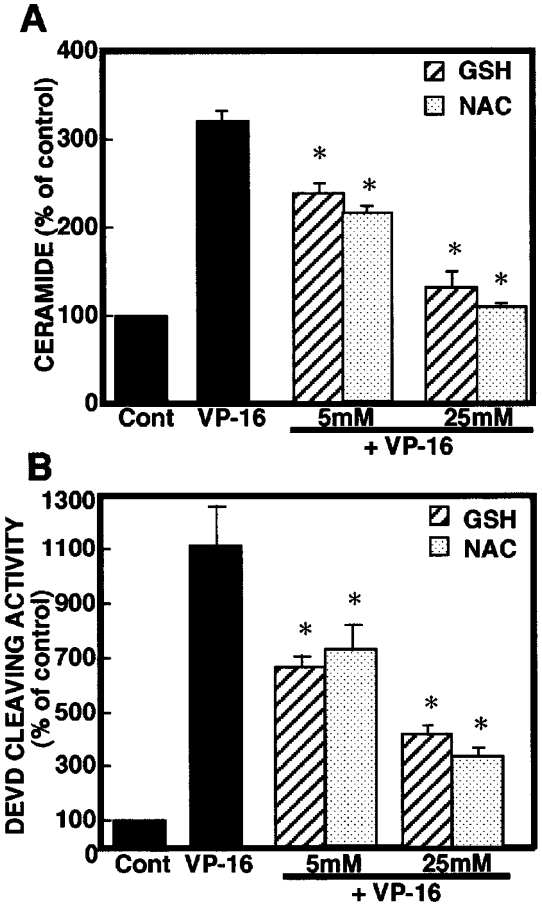

Figure 7 Effects of GSH or NAC on etoposide-induced ceramide formation and activation of caspase-3(-like) protease. C6 cells were preincubated with different concentrations of GSH or NAC for 1 day and then exposed to 40 μM etoposide (VP-16) for 24 h. (**A**) Ceramide content in C6 cells treated with etoposide in combination with or without GSH or NAC. Ceramide content was measured by the *E. coli* diacylglycerol kinase as described in Materials and Methods. (**B**) Activation of caspase-3(-like) protease in the presence or absence of GSH or NAC. Caspase-3(-like) activity was measured fluorometrically as described in Materials and Methods. Data are means $\pm$ S.D. from two independent experiments, each performed in triplicate. *$P<0.01$ versus VP-16 alone: two-way ANOVA followed by Scheffe's *post-hoc* test

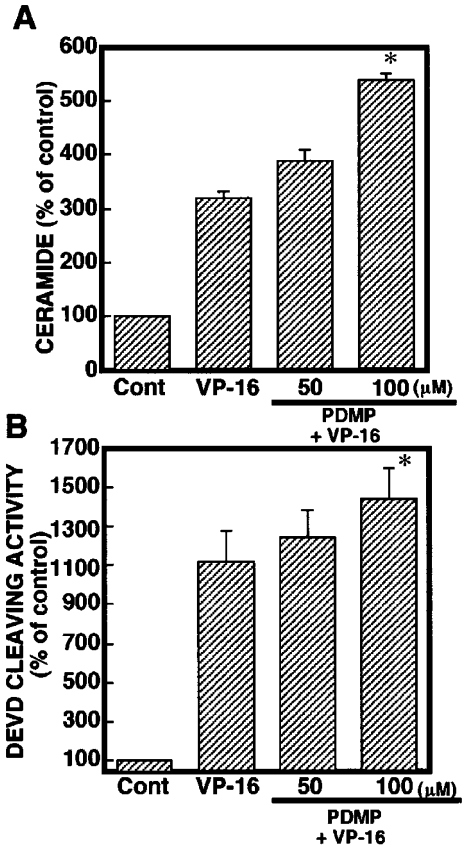

Figure 8 Effects of PDMP, an inhibitor of GlcT-1, on etoposide-induced ceramide formation and activation of caspase-3(-like) protease. C6 cells were preincubated with different concentrations of PDMP for 1 h and then exposed to 40 μM etoposide (VP-16) for 24 h. (**A**) Ceramide content in C6 cells treated with etoposide in the presence or absence of PDMP. Ceramide content was measured by the *E. coli* diacylglycerol kinase assay. (**B**) Activation of caspase-3(-like) protease in the presence or absence of PDMP. Caspase-3(-like) activity was measured fluorometrically. Data are means $\pm$ S.D. from two independent experiments, each performed in triplicate. *$P<0.01$ *versus* VP-16 alone: ANOVA with Scheffe's *post-hoc test*

ceramide formation by either 25 mM GSH or NAC prevented the etoposide-induced downregulation of Bcl-2 concomitant with upregulation of Bax (Figure 9B). In contrast, enhancement of ceramide formation by 100 μM PDMP significantly enhanced increase in the Bax/Bcl-2 ratio induced by etoposide (Figure 9C). These findings suggest that ceramide acts upstream of Bcl-2 and Bax.

Regulation of Bax/Bcl-2 expression and caspase activation by exogenous ceramide

In order to further confirm the role of ceramide in the regulation of Bax/Bcl-2 expression and caspase activation, we examined the effects of cell-permeable ceramide analogs, C_2- and C_6-ceramide, which both effectively induced apoptosis of C6 glioma cells.[18] At 12 and 24 h after treatment with 25 μM C_2-ceramide and 50 μM C_6-ceramide, respectively, approximately 80% cells became apoptotic (data not shown). Although Bax level was not significantly increased during the time course examined, C_2- and C_6-ceramide caused decrease in Bcl-2 (Figure 10A,B), thereby increase in Bax/Bcl-2 ratio. Processing of the initiator caspase-9 was first observed at 6 h after C_2-ceramide treatment, and the proform was almost completely processed at 9 h. Following caspase-9 activation, the levels of 32-kDa procaspase-3 began to be degradated at 9 h (Figure 10C). Therefore, the data obtained from experiments using exogenous ceramide support the notion that ceramide induces change of the Bax/Bcl-2 ratio due to the decrease of Bcl-2 and thereby activation of caspases in C6 glioma cells.

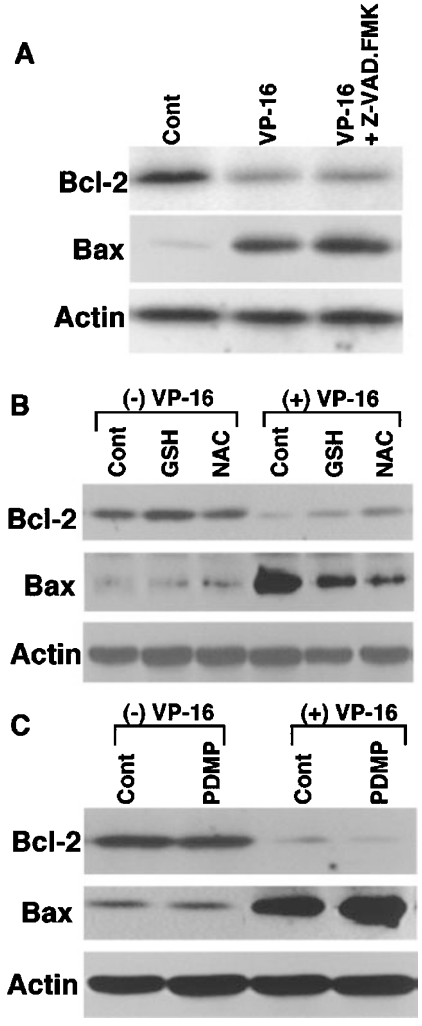

Figure 9 Effects of a caspase inhibitor, GSH or NAC, and PDMP on Bcl-2 and Bax expression induced by etoposide. C6 cells were preincubated with z-VAD·FMK, GSH or NAC, or PDMP, before exposure to 40 μM etoposide (VP-16) for 24 h. (**A**) Effect of 200 μM z-VAD·FMK. (**B**) Effect of 25 mM GSH or 25 mM NAC. (**C**) Effect of 100 μM PDMP. Cellular proteins were separated by SDS–PAGE and immunoblotted with antibodies against Bcl-2, Bax or actin as described in Materials and Methods. Results are representative of two separate experiments with compatible outcomes

Discussion

A number of recent studies indicate that caspases are closely involved in the initiation and execution of apoptosis induced by various stimuli.[20,21] Among them, caspase-3 has been deemed as an executor caspase. In etoposide-treated C6 cells, caspase-3 also takes a part in cell death, since a caspase inhibitor significantly decreased the apoptotic cells. Moreover, the number of apoptotic cells well correlated with the extent of caspase-3 activation, when etoposide treatment was modulated by a variety of agents. There are two main caspase cascades leading to executor caspase-3 activation.[22–30] One is initiated by caspase-8 activation associated with apoptosis induced by death receptors.[22–24] The other is initiated by caspase-9 activation which is triggered by cytochrome *c* release from mitochondria.[25–27] Two recent

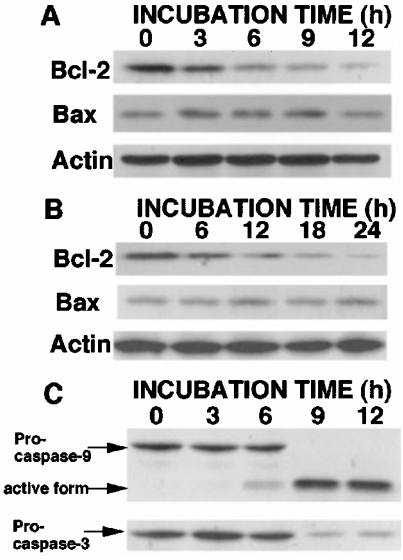

Figure 10 Exogenous ceramide-induced changes in protein levels of Bcl-2 and Bax, and processing of caspases-3 and -9 in C6 cells. After C6 cells were treated with 25 μM C_2-ceramide (**A**) or 50 μM C_6-ceramide (**B**) for indicated periods, cellular proteins were subjected to SDS–PAGE and immunoblotted with antibodies against Bcl-2 family as described in Materials and Methods. Cellular proteins from C6 cells exposed to 25 μM C_2-ceramide were separated by SDS–PAGE and immunoblotted with antibodies against caspases (**C**) as described in Materials and Methods. Results are representative of two separate experiments with compatible outcomes

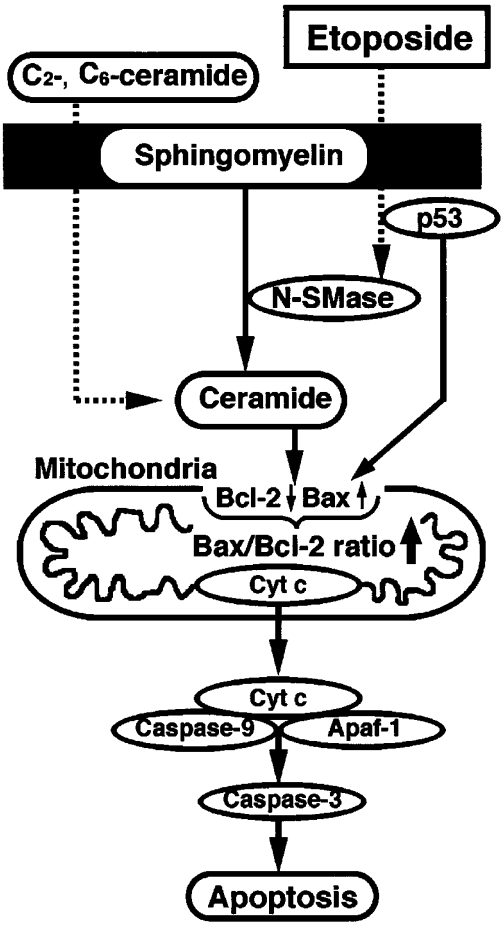

Figure 11 A hypothetical scheme of etoposide-induced apoptotic cell death of glioma cells. Etoposide induces activation of neutral SMase, which causes SM hydrolysis and production of ceramide. The ceramide accumulation triggers downregulation of Bcl-2 and may enhance, if any, the p53-dependent induction of Bax. This increase in Bax/Bcl-2 ratio leads to activation of caspases-9 and -3 through release of mitochondrial cytochrome *c*, thereby leading to apoptosis. Exogenous C_2- and C_6-ceramide mimic etoposide-induced ceramide pathway

studies on etoposide-induced apoptosis using Jurkat T cells[46] and human leukemia cells[47] have highlighted that procaspase-9 functions as an initiator caspase in the presence of dATP and cytochrome *c*. Its long N-terminal domain interacts with Apaf-1 resulting in processing and activation of caspase-9. Active caspase-9 then activates the effector caspase-3, thereby leading to PARP cleavage and DNA ladder. In agreement with these findings, our present study demonstrated that cytochrome *c* is released into the cytosol following the induction of apoptosis by etoposide and then caspases-9 and -3 are activated as initiator and effector caspases, respectively.

Ceramide has recently emerged as a novel second messenger for intracellular signaling pathways responding to various extracellular stimuli,[6–8] although the role of ceramide in apoptosis is still controversial.[13–17] Several lines of evidence indicate that ceramide is produced from SM by the action of endogenous neutral and/or acid SMase.[48] SM hydrolysis is shown to be induced not only by a variety of cytokines, including TNF-α, interferon-γ, interleukin-1β, nerve growth factor, and cross-linking of Fas, but also by physical stresses such as radiation and heat shock. To be noted, a chemotherapeutic agent has been demonstrated to induce formation of ceramide through SM hydrolysis.[35] It has been reported that ceramide plays an important role in apoptotic cell death pathway in glial and neuronal cells,[10–12,18] as observed in many types of cells.[7–9]

In the present study, etoposide caused accumulation of ceramide by activation of neutral SMase. Moreover, inhibition of etoposide-induced ceramide formation by GSH or NAC prevented apoptosis. In contrast, enhancement of ceramide formation by PDMP potentiated apoptosis. Furthermore, exogenous ceramide caused apoptosis, implying its apoptotic mediator role in C6 glioma cells. The inhibitors used (GSH, NAC, PDMP) may have other effects than we know and therefore each result cannot provide straightforward evidence regarding the role of ceramide. However, the combined weight of these data suggest that ceramide derived from SM hydrolysis functions as an important mediator of glial apoptosis induced by etoposide.

Lavie *et al*[49,50] demonstrated that multidrug-resistant human breast cancer cells exhibited marked accumulation of glucosylceramide compared with the parental cells. Tamoxifen, an agent that increases the sensitivity of multidrug-resistant cells to the toxic influence of previously less effective drugs, has been reported to inhibit GlcT-1, resulting in a decrease in the level of glucosylceramide with a reciprocal increase in the ceramide content. Therefore, these results suggest the possibility that regulation of ceramide levels may affect sensitivity of cancer cells to anticancer drugs.

The changes in the intracellular ceramide content by PDMP and GSH or NAC are positively correlated with etoposide-induced activation of caspase-3. Exogenous C_2- and C_6-ceramide caused apoptosis of C6 cell with activation of caspases-9 and -3. These results indicate that ceramide accumulation occurs upstream of caspase activation. Moreover, etoposide-induced ceramide accumulation was well correlated with changes of the Bax/Bcl-2 ratio. Therefore, it is tempting to speculate that ceramide modulates the activity of executor caspase-3 via changes of the Bax/Bcl-2 ratio and release of cytochrome *c*.

Recently, Dbaido *et al*[51] have demonstrated evidence that p53 may function upstream of ceramide accumulation in p53-dependent apoptotic pathways. On the other hand, p53 has been thought to differentially regulate two of the Bcl-2 family members that control apoptosis: Bax, an accelerator of apoptosis, is upregulated and Bcl-2, a suppressor of apoptosis, is downregulated.[39,41] In the bax promoter region, sequence-specific p53 binding sites are identified. DNA damaging agents including etoposide is known to induce expression of p53. We have observed induction of p53 expression in response to etoposide, but not to exogenous ceramide in C6 glioma cells (unpublished observation). Therefore, exogenous ceramide stimulated the decrease of Bcl-2 content but was unable to increase

Bax level. In other words, ceramide is the primary mediator of Bcl-2 downregulation, but may only act to upregulate Bax, if at all, in a p53-dependent manner. The possible mechanism of Bcl-2 reduction by ceramide has recently been reported by Schiavone *et al*[52] that ceramide destabilizes Bcl-2 mRNA via the adenine- and uracil-rich element (ARE).

GSH and NAC, known as inhibitors for neutral sphingomyelinase[42–44] and also reducing agents, inhibited changes of Bax/Bcl-2 ratio and caspase activation in response to etoposide. Although the exact molecular mechanism is not disclosed, p53-dependent formation of reactive oxygen species (ROS) has been demonstrated.[53–55] Li *et al*[55] has recently reported that this p53-dependent formation of ROS triggers the changes of mitochondrial membrane potential. Moreover, the recently cloned human neutral SMase is shown to be inhibited by GSH.[56,57] Indeed, Dbaido *et al*[51] have recently demonstrated evidence that p53 may function upstream of ceramide accumulation in p53-dependent apoptotic pathways. It is tempting to speculate that p53-dependent formation of ROS may reduce intracellular GSH level which turns neutral SMase on in etoposide-treated C6 glioma cells. Further studies are necessary to verify this hypothesis.

In summary, we would like to propose a hypothetical signaling sequence during etoposide-induced glial apoptosis (Figure 11). Etoposide causes accumulation of ceramide derived from SM hydrolysis by neutral SMase. The ceramide accumulation triggers downregulation of Bcl-2 and may enhance, if any, p53-dependent induction of Bax. This increase in Bax/Bcl-2 ratio leads to activation of caspases-9 and -3 through release of mitochondrial cytochrome *c*. Therefore, further understanding of the ceramide pathway, especially mechanisms of SMase activation and identification of direct targets of ceramide, during etoposide-induced apoptosis in glioma cells may give us a novel approach to treat malignant gliomas.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nipro (Osaka, Japan). Penicillin/streptomycin was from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was from Irvine Scientific (Santa Ana, CA, USA). Hoechst 33258 (bisbenzimide) staining dye, NAC, and Isogen were from Wako (Osaka, Japan). Immobilon-P membranes were from Millipore (Bedford, MA, USA). Reverse transcriptase was from Promega (Madison, WI, USA). *Taq* DNA polymerase was from Perkin Elmer Japan (Tokyo, Japan). The tetrapeptide substrates for caspase-1, Ac-DMQD-MCA, and for caspase-3, Ac-DEVD-MCA, and a protease inhibitor E-64 were obtained from Peptide Institute (Osaka, Japan). GSH and *Escherichia coli* diacylglycerol kinase were from Sigma (St. Louis, MO, USA). PDMP, an inhibitor of GlcT-1, C_2-, and C_6-ceramides were purchased from Matreya (Chalfont, PA, USA). [γ-^{32}P]ATP and ECL Western blotting detection reagents were from Amersham (Buckinghamshire, UK). [3-^{14}C]Serine (49 mCi/mmol) was from ICN Pharmaceuticals, Inc. (Irvine, CA, USA). High performance thin-layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). The broad spectrum caspase inhibitor, z-VAD·FMK was from Enzyme Systems Products (Dubin, CA, USA). Etoposide was supplied from Nippon-Kayaku (Tokyo, Japan). The primary antibodies used were anti-rat-Bcl-2 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse Bax polyclonal antibody (P-19: Santa Cruz Biotechnology), anti-rat ICE (procaspase-1) monoclonal antibody (Santa Cruz Biotechnology), anti-caspase-9 monoclonal antibody (Santa Cruz Biotechnology), anti-cytochrome *c* polyclonal antibody (Santa Cruz Biotechnology), and anti-mouse actin monoclonal antibody (Calbiochem-Novabiochem International). BCA protein assay reagents were obtained from Pierce (Rockford, IL, USA). Other chemicals were of the highest quality available.

Cell culture

C6 cells and U87-MG cells were obtained from Human Science Research Resources Bank (Osaka, Japan). The cells were maintained in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Prior to treatment with etoposide, C_2-, and C_6-ceramide, the cells were plated at a density of 5×10^4/ml and incubated for 2 days. For treatment with C_2- and C_6-ceramide, cells were then rinsed twice with phosphate-buffered saline (PBS) and cultured in DMEM medium without FBS for 24 h. Then, 25 μM C_2-ceramide or 50 μM C_6-ceramide was delivered into the culture medium without FBS.[18] For preincubation with z-VAD·FMK or PDMP, these agents were delivered in the medium 1 h before exposure to etoposide. Preincubation with GSH or NAC was performed for 1 day before etoposide treatment. Adequate concentration of each agent was determined from dose-response curves *versus* the extent of cell death (data not shown). Etoposide was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored at room temperature. It was diluted in the complete medium before each experiment.

Fluorescent microscopy

Apoptotic cells stained with Hoechst 33258 were quantified by fluorescent microscopic analysis.[58] Briefly, cells treated with etoposide were harvested and fixed in 1% glutaraldehyde for 30 min. The cells were rinsed with PBS and stained with 10 μM Hoechst 33258 for 10 min. Nuclear morphology was observed under a fluorescent microscope (Olympus BX60, Tokyo, Japan).

Measurement of cellular ceramide level by diacylglycerol kinase assay

Cellular ceramide level was measured as previously described.[58] Briefly, lipids extracted from cells were first treated in 0.1 M KOH in chloroform:methanol (1:2, v/v) at 37°C for 1 h. Ceramide was converted to ceramide 1-[^{32}P]phosphate by *E. coli* diacylglycerol kinase in the presence of [γ-^{32}P]ATP, and then lipids were separated on HPTLC plates in a solvent system of chloroform:acetone:methanol:acetic acid:water (50:20:15:10:5, v/v). Following autoradiography, spots corresponding to ceramide 1-phosphate were scraped into vials and the radioactivity was counted in a scintillation counter (Beckman LS-6500). Quantitation of ceramide was based on a standard curve of known amounts of ceramide. The changes in ceramide content were normalized based on total protein.

Measurement of [^{14}C]ceramide and [^{14}C]SM in [^{14}C]serine-labeled cells

To label sphingolipid, C6 cells (5×10^5 cells/10 ml) were cultured for 72 h in the medium containing 25 μCi of [^{14}C]serine. Lipids were extracted as described previously[58] and the lower phase was collected and evaporated under a flow of nitrogen gas. The extracted lipids were incubated in 0.1 M KOH in chloroform:methanol (1 : 2, v/v) at 37°C for 1 h. Samples were then separated on HPTLC plates using the solvent system of chloroform : methanol : water (70 : 30 : 50, v/v) for SM or chloroform : methanol (95 : 5, v/v) for ceramide. Following autoradiography, spots were scraped and the radioactivity was determined by liquid scintillation counter (Beckman LS-6500). The changes in ceramide content were normalized based on total protein.

SMase assay

Membrane and cytosolic fractions were prepared from cells after exposure to etoposide. Briefly, cells were washed in ice-cold PBS and homogenized in lysis buffer (25 mM Tris/HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml E-64) with 20 strokes in a glass homogenizer with a Teflon pestle (Iuchiseieido, Tokyo, Japan). The post-nuclear homogenate was centrifuged at 100 000 $\times$ *g* for 1 h and the pellet was resuspended in lysis buffer. The activities of both neutral and acid SMases were determined using a mixed micelle assay system as described.[59] For determining neutral SMase activity, the membrane fractions (20 μg protein) were mixed with [*methyl*-^{14}C]SM (40 000 c.p.m. in 1 nmol of bovine brain SM in 0.25% Triton X-100 solubilized by sonication) in 0.1 M Tris/HCl buffer (pH 7.4) containing 6 mM $MgCl_2$. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 1.5 ml of chloroform : methanol (2 : 1, v/v) followed by 0.2 ml of H_2O. After phase separation, a portion of the upper phase was transferred to scintillation vials and the radioactivity was determined by liquid scintillation counting. Negative controls containing no enzyme was run concomitantly. Acid SMase activity in membrane was measured as above except that the Tris/HCl buffer was replaced with 0.1 M sodium acetate buffer (pH 5.5) containing 5 mM EDTA.

Activity of caspase proteases

Cells were harvested after exposure to etoposide for indicated periods of time and washed three times with PBS, and then suspended in buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA and 10 mM EGTA. After addition of 10 μM digitonin, cells were incubated at 37°C for 10 min. Lysates were centrifuged at 900 $\times$ *g* for 3 min, and the resulting supernatant (40 μg protein) was incubated with 50 μM of enzyme substrate at 37°C for 1 h. Levels of released 7-amino-4-methylcoumarin (AMC) were measured using spectrofluorometers (Hitachi F-3000 and F-2000, Japan) with excitation at 380 nm and emission at 460 nm.[56] Excitation and emission slit widths were adjusted to 10 and 20 mm, respectively. One unit was defined as the amount of enzyme required to release 0.22 nmol AMC per min at 37°C.

RNA extraction and reverse transcription-polymerase chain reaction (RT – PCR)

Total RNA was isolated from C6 cells with Isogen, according to the manufacturer's instruction. RNA (2 μg) was reverse transcribed using random hexamer-mixed primers. Primer sets for rat *bcl-2* and *bax* were newly designed; 5′-GAACACCAGAATCAAGTGTTCG-3′ (sense) and 5′-TCAGGTGGACCACAGGTGGC-3′ (antisense) for *bcl-2*,[59] and 5′-AGGGTTTCATCCAGGATCGAGC-3′ (sense) and 5′-AGGCGGTGAGGACTCCAGCC-3′ (antisense) for *bax*.[60] The PCR primer set for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was prepared according to the previous report.[61] The temperatures used for PCR were as follows: denaturation at 94°C for 30 s; annealing at 58°C for 1 min; and extension 72°C for 1 min. The numbers of amplification cycles were determined to individual primer sets in order to maintain exponential rate of product amplification. The number of cycles determined for GAPDH was 22, for *bax* was 26, and for *bcl-2* was 30, respectively. Amplified DNA fragments were subjected to electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining. The intensity of bands was quantified by a densitometer (Atto Densitograph, Tokyo, Japan).

Preparation of cytosolic fraction for measurement of cytochrome *c* release

Cytosolic extracts were prepared according to the previous report.[29] Briefly, cells were collected at the indicated times and washed twice with ice-cold PBS and resuspended in 100 μl of extraction buffer (50 mM HEPES-KOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmetylsulfonyl fluoride, and 1 mM E-64). After incubation on ice for 20 min, cells were homogenized with 20 strokes in a glass homogenizer with a Teflon pestle. Nuclei were removed by centrifugation at 1000 $\times$ *g* for 10 min at 4°C in a microcentrifuge. Supernatants were then further centrifuged at 10 000 $\times$ *g* for 15 min at 4°C. The supernatant was further clarified by centrifugation at 100 000 $\times$ *g* for 60 min at 4°C.

Western blot analysis

For the analysis of processing of procaspases, cells were solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml E-64. Insoluble materials were removed by centrifugation at 1000 $\times$ *g* for 10 min at 4°C in a microcentrifuge. Supernatants were then further centrifuged at 10 000 $\times$ *g* for 15 min at 4°C. The supernatant was further clarified by centrifugation at 100 000 $\times$ *g* for 60 min at 4°C.

Western blot analysis

For the analysis of processing of procaspases, cells were solubilized with ice-cold lysis buffer containing 1% Triton X-100, 50 mM NaCl, 25 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml E-64. Insoluble materials were removed by centrifugation at 10 000 $\times$ *g* for 10 min. Extracted proteins (60 μg/well) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS – PAGE) on 13% polyacrylamide gels, and were electrophoretically transferred onto Immobilon-P membrane. Blocking was performed in Tris-buffered saline containing 5% skimmed-milk powder and 0.1% Tween-20. The membranes were probed with antibodies against CPP32 (procaspase-3), ICE (procaspase-1), caspase-9, cytochrome *c*, Bcl-2, Bax, or actin proteins. Detection was performed with ECL system. Protein content was determined with BCA protein assay using bovine serum albumin as a standard.

Statistical analysis

Data are expressed as means $\pm$ S.D. Significance was assessed by one-way or two-way ANOVA, followed by Scheffe's *post-hoc* test. *P* values less than 0.01 was considered as significant.

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