Potential Mechanisms Involved in Ceramide-induced Apoptosis in Human Colon Cancer HT29 Cells[1]

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Objective To investigate the potential mechanisms of cell death after the treatment with ceramide. **Methods** MTT assay, DNA ladder, reporter assay, FACS and Western blot assay were employed to investigate the potential mechanisms of cell death after the treatment with C2-ceramide. **Results** A short-time treatment with C2-ceramide induced cell death, which was associated with p38 MAP kinase activation, but had no links with typical caspase activation or PARP degradation. Rather than caspase inhibitor, Inhibitor of p38 MAP kinase blocked cell death induced by a short-time treatment with ceramide (<12 h). However, inhibition of p38 MAP kinase could not block cell death induced by a prolonged treatment with ceramide (>12 h). Moreover, incubation of cells with ceramide for a long time (>12 h) increased subG1, but reduced S phase accompanied by caspase-dependent and caspase-independent changes including NFκB activation. **Conclusion** Ceramide-induced cell apoptosis involves both caspase-dependent and -independent signaling pathway. Caspase-independent cell death occurring in a relatively early stage, which is mediated via p38 MAP kinase, can progress into a stage involving both caspase-dependent and –independent mechanisms accompanied by cell signaling of MAPKs and NFκB.

Key words: Ceramide; HT29 cells; Apoptosis; Cell signaling; p38 MAPK; NFκB

INTRODUCTION

Ceramide, a metabolite of sphingolipids generated by degradation of sphingomyelin through the action of sphingomyelinases, condensation of sphinganine or sphingosine and fatty acyl-CoA via ceramide synthesis, has emerged as a novel lipid second messenger with a specific role in cell signaling implicated in cell growth, proliferation, apoptosis and other cell responses[1]. The biological effects of ceramide depends on many parameters, such as cell type, nature of cell receptors and their concentration, suggesting the existence of multiple downstream targets activated through distinct intracellular pathways. C2-ceramide, a non-natural but cell-permeable analog of the endogenous long-chain ceramides, has been used in a variety of studies to display the biological effects of the endogenous ceramide.

One of the most clearly illustrated targets of ceramide is cell death[2-5]. Many factors for apoptosis, including TNF-α, CD95/Fas/APO-1, serum deprivation, chemotherapeutic drugs, radiations, heat shock, and oxidative stress, appear to induce a rapid rise of intracellular ceramide concentration by regulating one or more enzymes of ceramide metabolism[6-7]. Interestingly, ceramide not only plays an important role in one or several stages of apoptosis, but also in caspase-independent and/or non-apoptotic cell death. Although the main biological function of ceramide appears to be linked to its potency to induce cell death, its actual relevance as a regulator of cell death is controversial.

The implications of JNK and p38 MAPK in ceramide-induced cell death in various cell types have been revealed by pharmacological and molecular assays[8-10]. However, it has also been demonstrated that p38 MAPK may not be essential for ceramide-induced cell death in U937 and MC/9 cells[11-12]. On the other hand, it has been reported that NFκB activation is involved in apoptosis inhibition[13-14]. However, consisting activation of NFκB could also induce

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pre-apoptotic gene expression in some neuron cells[15-16]. Indeed, the mechanism of ceramide-induced apoptosis is still controversial and various hypotheses have been postulated[17-19].

We have previously found some data about MAPK signaling in apoptotic HT29 cells induced by C2-ceramide[20]. However, neither MAPK nor NFκB signaling has been investigated in details. The present study is designed to examine the potential mechanisms of HT29 cell death induced by C2-ceramide treatment. Our data demonstrate that ceramide-induced apoptotic cell death involves both caspase-dependent and -independent signaling pathway. Caspase-independent cell death occurring in an early stage activated by p38 MAPK can progress into a stage involving both caspase-dependent and -independent mechanism accompanied with cell signaling of MAPKs and NFκB.

MATERIALS AND METHODS

Reagents

C2-ceramide was purchased from Sigma Co. PD98059, SB203580 and zVAD-fmk were from Alexis Biochemical. Anti-NFκB antibody was from Cell Signaling. Anti-CDK7, procaspase-3 (32kDa), cleaved caspase-3 (17kDa) and β-actin antibodies were from Santa Cruz, Inc. Anti-phosphorylated-ERK, JNK, p38 antibodies were from New England Biolabs, Inc. Anti-cleaved PARP antibody was from Abcam. MTT, propiudium iodide (PI), IκB inhibitor PDTC and other chemicals were all from Sigma.

Cell Culture and Treatment

HT29 cells (originally from ATCC) obtained from the Chinese Academy of Medical Sciences were maintained in Dulbecco's minimal essential medium supplemented with 10% FBS. Subculture of cells was performed by enzymatic digestion (trypsin / EDTA solution: 0.25%/0.02%). C2-ceramide and other reagents dissolved in ethanol or dimethyl sulfoxide (DMSO) were used without filtration. Final concentration of DMSO or ethanol in culture medium was < 0.3%.

Determination of Cell Death

Cell viability was ascertained by MTT assay (20 μL of 5 mg/mL MTT in PBS) and the absorbance was measured at 570 nm.

Flow Cytometric Analysis for Apoptosis

(1) PI staining: Cells were collected after treatment and washed 3 times with PBS, incubated with PBS containing 100 μg/mL propidium iodide (PI), 1%Triton X-100, 100 U/mL RNase for 30 min at 37 ℃, followed by FACS analysis.

(2) Annexin V FITC labeling: Phosphatidylserine (PS) expression on the external surface of cells was detected in terms of binding to FITC-labeled annexin V. Cells were collected and washed once with cold PBS, and centrifuged to collect the cell pellet which was resuspended in a cold binding buffer (10 mmol/L HEPES/NaOH, pH 7.4, 140 mmol/L NaCl, 5 mmol/L $CaCl_2$). Annexin V-FITC (final concentration 1 μg/mL) was added and mixed gently. The tubes were then incubated for 15-20 min in the dark prior to flow cytometry.

DNA Ladder Assays

Cells were cultured with or without C2-ceramide (30 μmol/L) in the presence or absence of zVAD-fmk and p38 MAPK inhibitor SB203580 for 24 h. Genomic DNA was extracted and resolved on 1.5% agarose gel. Cells were collected and suspended in a lysis buffer containing 10 mmol/L Tris·Cl (pH8.0), 10 mmol/L NaCl, 10 mmol/L EDTA, 100 μg/mL proteinase K, 1% SDS, and incubated at 37 ℃ for 4 h. Lysate was extracted 3 times with phenol: choloroform (1:1, V: V). DNA was precipitated with 1/10 volume of 3 mol/L sodium acetate (pH 4.0) and 2 volumes of ethanol. The cell pellet was dissolved in 20 μL TE with 2 mg/mL RNaseA and subjected to electrophoresis on 1.5% agarose gel.

Western Blot

Cell lysate in a Laemmli reducing sample buffer was separated on 12% (for p-JNK, p-ERK, p-p38, p38, p65 NFκB and actin) or 6% (for cleaved PARP) SDS-PAGE. Total proteins were transferred onto PVDF membrane after electrophoresis. Western blot assay was performed using specific antibody.

Immunocytochemistry by Confocal Microscopy

Cells were seeded on coverlips into six-well culture dishes and incubated in DMEM with or without C2-ceramide. The coverlips were washed in PBS, fixed in methanol: acetone (1:1, v/v) for 30 min and stained with primary antibody recognizing p65NFκB for 1 h. After washing, the glasses were incubated for 30 min with secondary antibody labeled with FITC. Cells were examined under a laser scanning confocal microscope (Zeiss, LSM 510).

Transient Transfection and Luciferase Assay

HT29 cells were seeded at a concentration of 1×10^5 cells/35 mm dish. After 12 h, complete medium was replaced with DMEM without FBS or antibiotic. Transfection was done using Lipofectamine reagent (Gibco BRL) mixed with pNFκB-luc and the control vector SV40 for 8 h. After the transfection mixture was replaced with a medium containing 2% FBS, cells

were incubated for 2 h with or without the inhibitor of the release of IκB (inhibitor κB) from the cytoplasmic IκB-NFκB complex, PDTC (5 μmol/L) and then with or without tested chemicals for further 24 h. Luciferase activity was measured according to the manufacturer's protocol (Promega).

RESULTS

Ceramide Induced Apoptosis in HT29 Cells

Treatment with different concentrations of C2, C6, C8-ceramides for 24 h exhibited significant cytotoxicity against HT29 cells as suggested by MTT assay (Fig. 1A). Microscopy also showed proliferation inhibition when HT29 cells were treated with C2-ceramide for different periods of time (Fig.1B). The cytotoxicity was derived from its ability to induce apoptosis, which was demonstrated by HE staining (Fig. 1C), formation of apoptotic bodies (Fig.1D) and percentage of SubG1 phase (Fig. 1E), respectively. Further investigation showed that C2-ceramide treatment could effectively inhibit the expression of CDK7 protein (Fig. 1F).

Ceramide Induced Apoptosis via Caspase-dependent and -independent Pathway

Cell cycle distribution of HT29 cells treated with C2-ceramide for different periods of time was shown by the accumulation of percentage of SubG1 phase and decrease in S phase (Table 1). However, the accumulated percentage of SubG1 phase in the earlier stage of treatment was much less than that in the late stage, which was verified by the percentage of apoptosis determined by annexin V staining (Fig. 2A). Determination of the cleaved PARP following C2-ceramide treatment for different periods of time as shown in Fig. 2B indicated that C2-ceramide mediated enhanced PARP cleavage in the relatively late stage (more than 12 h) rather than in the earlier stage (less than 12 h), which was presumably attributed to enhanced caspase activity. To test directly whether caspase activation is involved in the late stage, HT29 cells were pretreated with pan-caspase inhibitor, zVAD-fmk and then with C2-ceramide, demonstrating that zVAD-fmk effectively protected HT29 cells from apoptosis suggested by annexinV-PI staining (Fig. 2C) and DNA laddering (Fig. 2D) in the relatively late stage. The finding of Western blot assay to detect real active caspase-3 also indicated that the caspase pathway was involved (Fig. 2E). However, other than the caspase-dependent pathway, the caspase-independent pathway was also involved in the relatively earlier stage (Fig. 2C, D and E).

TABLE 1

Effect of 30 μmol/L Ceramide on the Cell cycle Distribution in HT29 Cells

Treatment	Sub G_1 (%)	S phase (%)
Control (0 h)	1.32	16.34
2h	4.35	12.06
4h	7.08	11.45
6h	5.98	11.87
8h	11.64	8.27
12h	19.86	8.62
20h	35.21	9.14
24h	54.06	4.93

MAPK Signaling in Ceramide-induced Apoptosis

Involvement of MAP kinases in apoptosis of different cell types has been identified[21-22]. However, it is still controversial[23-24]. MAPK pathway was evaluated in this study. Cell extracts were prepared from HT29 cells treated with C2-ceramide for different periods of time. As shown in Fig. 3A and Fig. 3B, p38 MAP kinase was activated in the relatively early stage (less than 12 h), but was not so in the later stage (more than 12 h). JNK was activated in the later stage, while ERK kept unchanged in the whole process, which was confirmed by the morphological change of HT29 cells pretreated with specific inhibitors, SB203580 and PD98059, respectively (Fig. 3C). Inhibition of p38, instead of ERK MAPK , could prevent the apoptosis induced by C2-ceramide treatment for 8 h (Fig. 3C). Nevertheless, in the relatively late stage (>12 h), inhibition of p38 MAPK by SB203580 was unable to inhibit the cell death induced by a long-term treatment with ceramide (24 h), which was also demonstrated by the DNA laddering data (Fig. 2D).

NFκB Signaling in Ceramide-induced Apoptosis of HT29 Cells

Nuclear factor kappa B (NFκB) has been proposed as an anti-apoptotic factor, which plays a key role in cell survival and angiogenesis. However, there are also controversial reports. Therefore, we investigated the effect of ceramide on the activation of NFκB by Western blot assay and confocal microscopy, respectively. The results showed that p65 NFκB expression increased after the treatment with C2 and C8-ceramides for 24 h (Fig. 4A). The effect of C2-ceramide on the localization distribution of NFκB was studied using an indirect immunofluorescence assay and confocal microscopy. Control cells showed a resulting immunoreaction localized in cytoplasm of the cells. On the contrary, treatment with 30 μmol/L C2-ceramide for 24 h led to pronounced nuclear translocation of NFκB. At the same time, nuclear structure was altered, accompanied

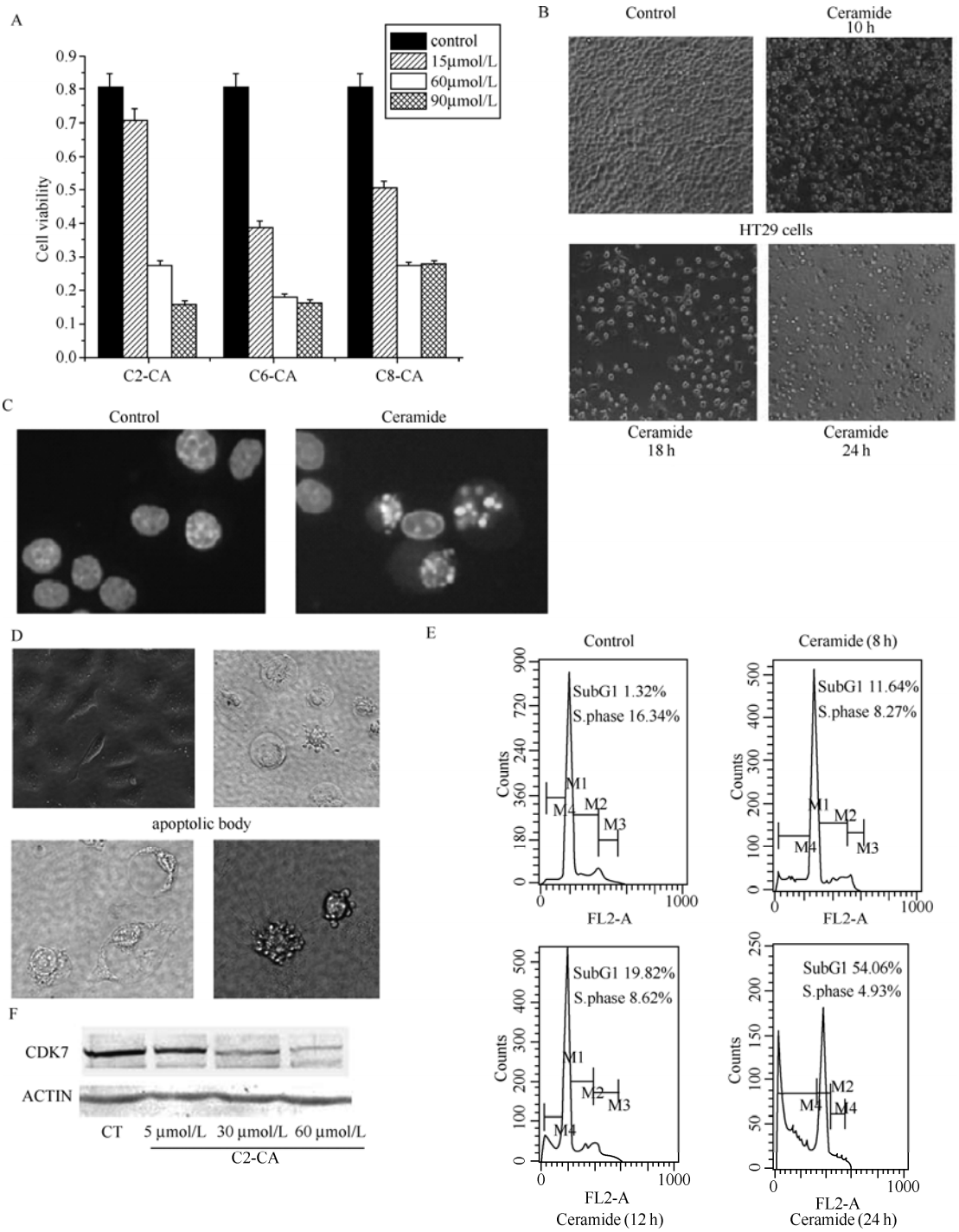


FIG. 1. Ceramide induces apoptosis in HT29 cells. A: Cells were cultured in a medium with different concentration of C2, C6, C8-ceramides for 24 h. Cell viability was analyzed by MTT assay. B: Cells were cultured with C2-ceramide (30 μmol/L) for different periods of time. Proliferation inhibition was shown by microscopy (×200). C and D: Cells were cultured with or without C2-ceramide (30 μmol/L). Apoptosis was demonstrated by HE staining method (×400) (C). Apoptotic body was observed by microscopy (×400) (D). E: Cells were cultured with C2-ceramide (30 μmol/L) for different periods of time. Apoptosis was detected by FACS assay with PI staining. F: Cells were cultured with different concentration of C2-ceramide for 24 h. CDK7 protein levels were measured by Western blot using anti-CDK7 antibody. The data were expressed as e $\bar{x} \pm s$ (n=3-4).

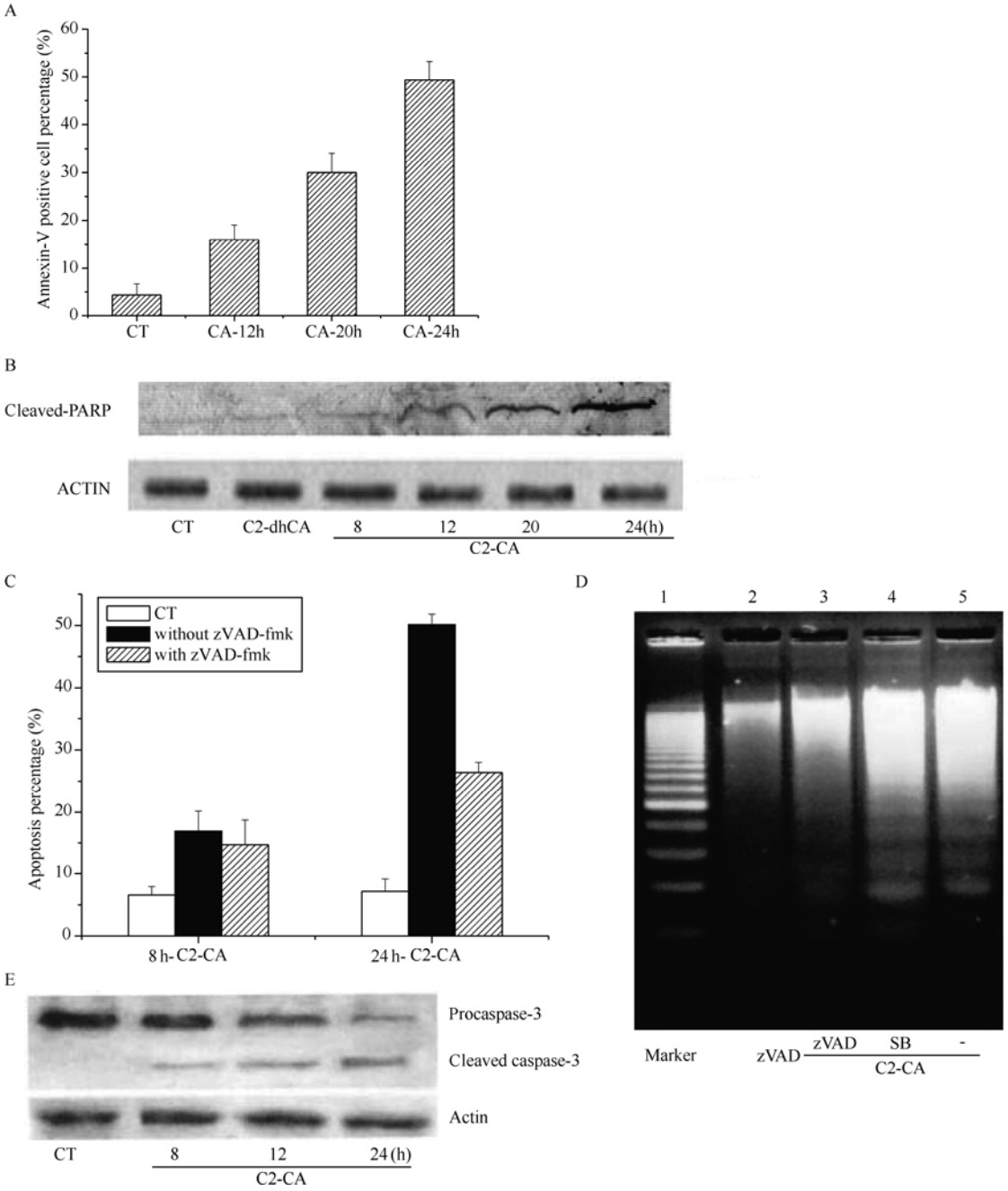


FIG. 2. Ceramide induces apoptosis *via* caspase-dependent and caspase-independent pathway. A: HT29 cells were cultured in a medium with C2-ceramide (30 μmol/L) for different periods of time as indicated. Apoptosis was detected by FACS assay with annexin V staining. The bar represents the relative number of apoptotic cells stained with annexin V. B: Cells were cultured in the medium with C2-ceramide (30 μmol/L) for different periods of time as indicated. Cleaved-PARP protein level was measured by Western blot using anti-cleaved-PARP antibody. C: Cells were cultured with or without C2-ceramide (30 μmol/L) in the presence or absence of zVAD-fmk for different periods of time as indicated. Apoptosis was detected by FACS assay with annexin V staining. The bar represents the relative number of apoptotic cells stained with annexin V. The data were expressed as mean ± SE (n=3-4). D: Cells were cultured with or without C2-ceramide (30 μmol/L) in the presence or absence of zVAD-fmk and p38 MAPK inhibitor SB203580 for 24 h. Genomic DNA was extracted and resolved on 1.5% agarose gel. E: Cells were cultured in amedium with C2-ceramide (30 μmol/L) for different periods of time as indicated. Procaspase-3 and cleaved caspase-3 levels were measured by Western blot using specific antibody.

with condensed chromatin, a phenomena indicating apoptosis (Fig. 4B), which was confirmed by Western blot assay of different parts of cells treated with C2-ceramide for 24 h (Fig. 4B, bottom line). The results of the reported assay indicated that NFκB was activated when cells were treated with C2-ceramide, which was down-regulated when cells were pretreated with PDTC, an inhibitor of κB from the cytoplasmic IκB-NFκB complex (Fig. 4C). As detected by MTT assay, the cytotoxicity of HT29 cells was enhanced when cells were pretreated with PDTC, followed by the treatment with C2-ceramide. The cytotoxicity in the relatively late stage was displayed in a dose- and time-dependent manner (Fig. 4D and Fig. 4E).

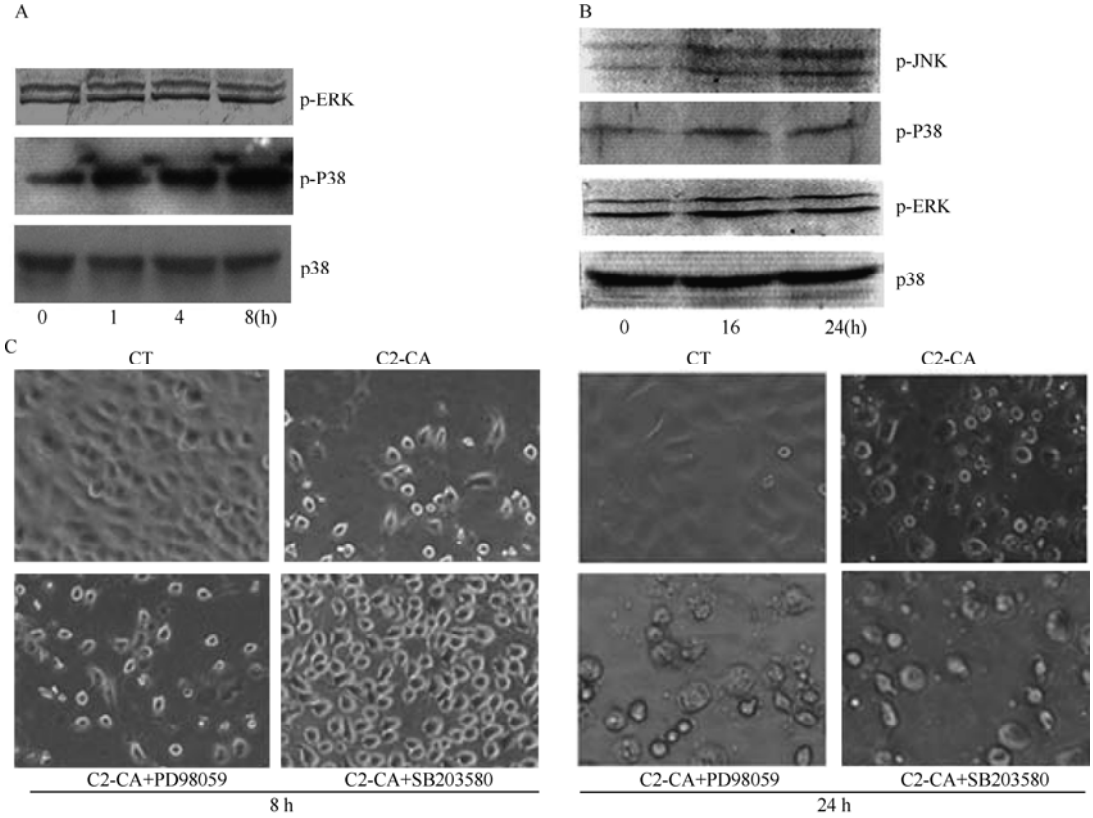
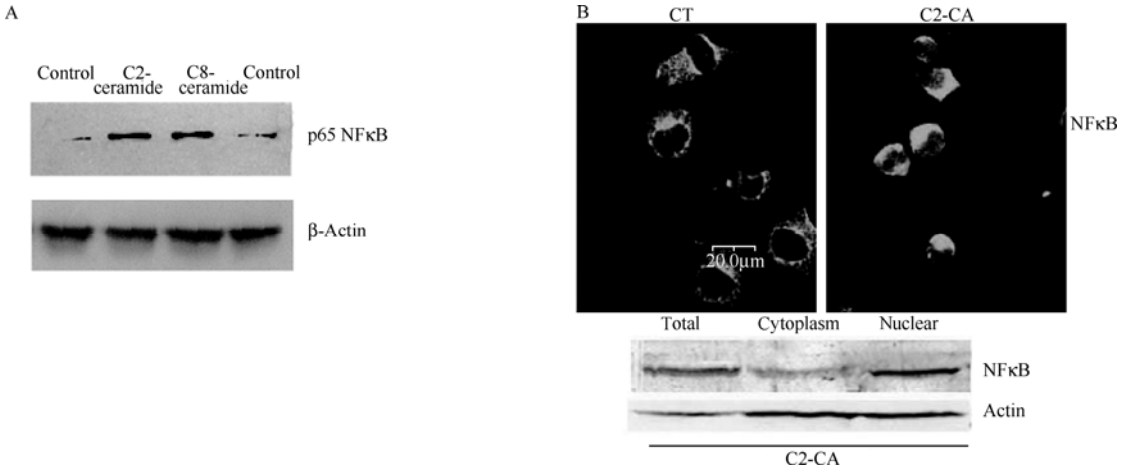


FIG. 3. MAPK signaling in ceramide-induce apoptosis of HT29 cells. A and B: Cells were cultured in the medium with C2-ceramide (30 μmol/L) for different periods of time as indicated. Total proteins were extracted and resolved on SDS-PAGE followed by Western blot assay using anti-phospho JNK, ERK and p38 antibodies. Data shown in Fig. 3A are in the relatively earlier stage (0, 1, 4, 8(h)), data that the JNK was not activated were not shown, and data shown in Fig. 3B are in the late stage, which are more than 12 h (0, 16, 24(h)). C: Cells were pretreated with PD98059 and SB203580 for 30 min, respectively, and cultured in the medium with 30 μmol/L of C2-ceramide for 8 and 24 h, respectively. Morphological changes of cells were then observed under microscope (×400).



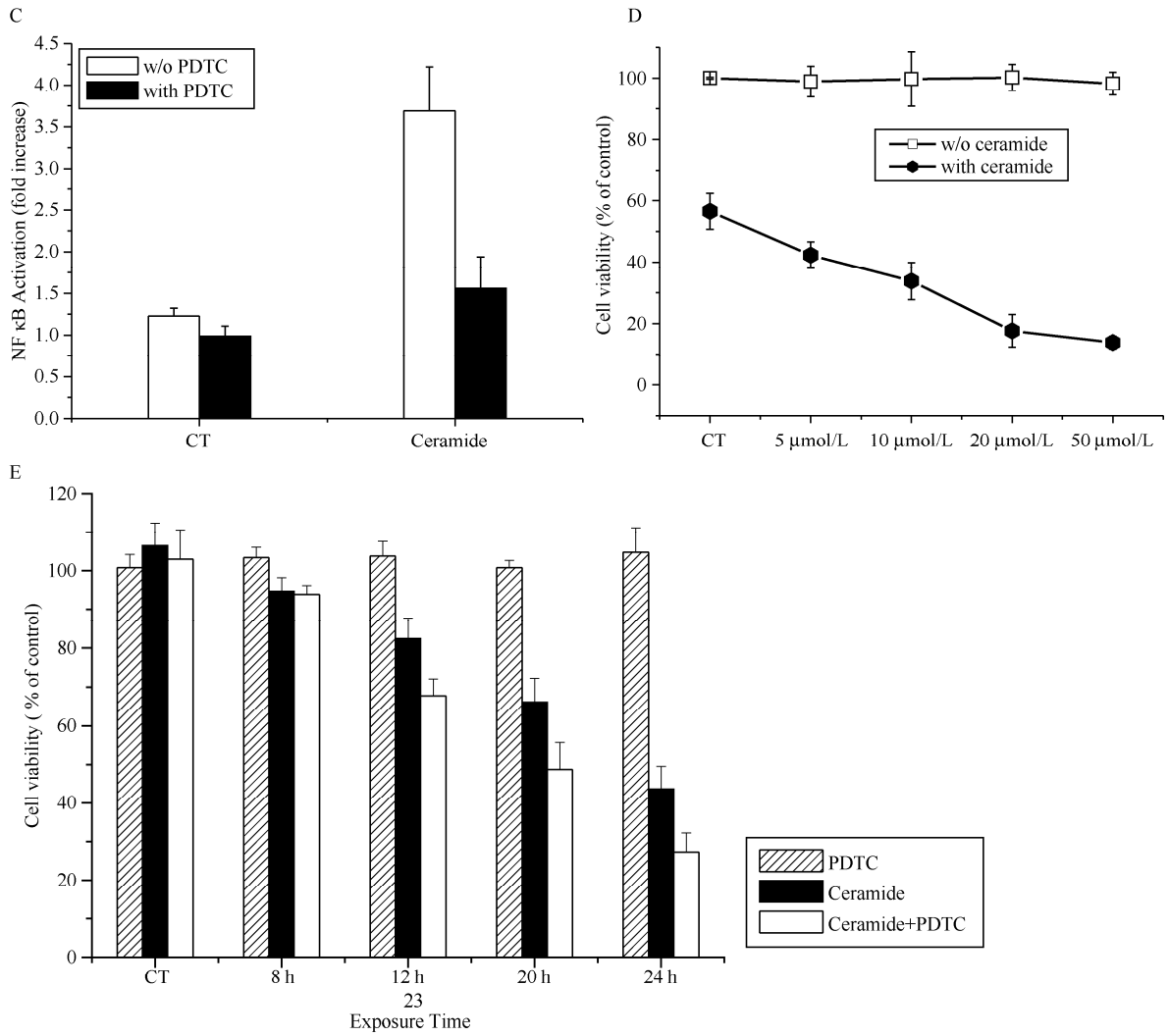


FIG. 4. NFκB involved in ceramide-induced apoptosis of HT29 cells. A: Cells were cultured with or without 30 μmol/L of C2, C8-ceramide for 24 h. p65 NFκB fragment protein level was measured by Western blot using anti- p65 NFκB and β-actin antibodies. B: Cells were cultured with or without 30 μmol/L of C2-ceramide for 24 h. NFκB translocation from cytoplasm to nuclear was observed under confocal microscope after cells were fixed and used for immunohistochemistry assay. C: Cells were pretreated with PDTC, cultured with 30 μmol/L of C2-ceramide for 24 h, transiently transfected with NFκB reporter or the control vector. NFκB transcriptional activity was measured as described. The bar represents the relative fold increase in luciferase activity. The data were expressed as $\bar{x} \pm s$ (n=3-4). D. Cells were cultured with 30 μmol/L of C2-ceramide in the presence or absence of PDTC with different concentrations for 24 h. Cell viability was determined by MTT assay. E: Cells were cultured with C2-ceramide in the presence or absence of PDTC (20 μmol/L) for different periods of time. Cell viability was determined by MTT assay. The data were expressed as $\bar{x} \pm s$ (n=3-4).

DISCUSSION

Apoptosis is mediated by various cellular events including protein synthesis and degradation, alteration in protein phosphorylation status, activation of lipid second messenger systems and disruption of mitochondria function. Recently, ceramide has shown its inhibitory effect on cell growth via apoptosis in a variety of cancers, since it was confirmed as an important lipid second messenger in 1994. However, its mechanism is not yet clearly understood. In this study, ceramide could induce caspase-dependent and -independent apoptosis involved in activation of p38 MAP kinase or NFκB, which was modulated by different mechanisms in different periods of treatment.

It has been shown that ceramide is able to activate a genetically regulated suicide program. Ceramide-induced cell death varies with cell types and cellular environment, demonstrating that cellular

context and environment may contribute to the types of cell death induced by ceramide. In our study, ceramide could induce apoptosis of HT29 cells, which was demonstrated by HE staining, formation of apoptotic bodies and accumulation of percentage of SubG1 phase, respectively. It has been reported that ceramide acts as a cell cycle suppressor that can lead to G1/G0 arrest[25-27]. In this study, S phase cells were decreased after the treatment with ceramide. Moreover, decrease in S phase cells is associated with an increase in sub-G1 DNA contents, indicating that DNA cleavage and caspase-dependent apoptosis are induced. It is reported that CDK7 in nuclei and cytoplasm is attached to DNA template with other TFH subunits initiating gene transcription[28], which is pivotal to modulate cell proliferation. In our study, C2-ceramide treatment obviously inhibits CDK7 expression, indicating that CDK7 alteration may contribute to the effect of C2-ceramide on cell cycle distribution alteration.

In the present study, caspase-independent apoptosis is induced by a short-time treatment with ceramide, followed by caspase-dependent and -independent apoptosis. The caspase-independent apoptosis occurring in the early stage could not be determined by classic phenotypes of apoptosis. However, in the relatively late stage (more than 12 h), C2-ceramide enhanced PARP cleavage and cleaved caspase-3, are presumably attributed to enhanced caspase activity, suggesting that caspase-dependent and -independent apoptosis occurs in the late stage.

Involvement of cell signaling is further investigated. Activation of MAP kinase pathway is involved in apoptosis of different cells, such as PC12 death induced by NGF withdrawal[21] and H_2O_2 stress[22] and T cell apoptosis induced by glucocorticoid[29]. In our experimental model, the activation of p38 and JNK is observed in HT29 cells treated with C2-ceramide (30 μmol/L) for different periods of time. Although ERK MAP kinase can be down-regulated by ceramide in some cell lines, our data have demonstrated that C2-ceramide fails to affect phosphoration of ERK and total ERK proteins (Fig. 3A and 3B). A number of studies have reported that p38 MAPK is involved in the mediation of ceramide-induced cell death[8-9,30-31]. Nevertheless, no report supports the role of p38 MAPK in the mediation of ceramide-induced apoptosis[11-12]. Our data show that C2-ceramide could activate p38 MAPK phosphoration, which is consistent with the reports that sphingomyelinase and ceramide activate p38 in 184B5/HER cells[32] and NCI-H292 cells[6]. In our study, inhibition of ERK activation by MEK inhibitor PD98059 has no effect on ceramide-induced cell death, whereas inhibition of p38 MAP kinase by specific inhibitor SB203580 may suppress ceramide-induced death, indicating that p38 activation plays an important role in the capase-independent cell death induced by a short time treatment with ceramide. However, neither PD98059 nor SB203580 could affect the cell death induced by a long-term treatment with ceramide. On the contrary, capase inhibitor effectively, though not entirely, inhibites the DNA fragmentation induced by a long-term treatment with ceramide and partially inhibites PARP degradation. These results demonstrate that ceramide may have two different potential mechanisms underlying the apoptosis of HT29 cells. Although the mechanism remains to be further studied, differential signaling pathways might be involved in the regulation of ceramide-induced apoptosis. Apoptosis induced by long-term ceramide treatment involves caspase-dependent and -independent events and is associated with cell cycle-related signaling events, whereas caspase -independent apotosis induced by short-term ceramide treatment is mediated via the mechanism by which p38 MAPK plays an important role.

On the other hand, nuclear factor kappa B has been proposed as an anti-apoptotic factor, which plays a key role in cell survival and angiogenesis. NFκB is a protein factor which can specifically bind to the enhancer κB, and the latter is activated following transcriptional activation of specific genes when cells are exposed to various stresses, such as inflammatory media, virus infection and oxygen stresses. Previous studies have shown that there are three major mechanisms involved in apoptosis inhibition induced by the activation of NFκB which can induce the expression of TRAF1/2 (tumor necrotic factor receptor associated factors 1 and 2) and cellular endogenous anti-apoptotic proteins cIAP1/cIAP2: TNF receptor signaling pathway, induced-expression of anti-apoptotic genes (Bcl-2 and Bcl-xL), and accumulation of the expression of MnSOD.

This study has demonstrated that ceramide could activate the expression and activity of NFκB. It has been reported that use of acid- and neutralized-sphingomyelinase could lead to significant activation of NFκB[33-34], which is consistent with our findings. Many specific inhibitors of the NFκB signaling pathway have been used, such as Bay11-7085, SCH66336, PDTC, and lactacystin in the treatment of cancer patients with apoptosis[35-38].

To gain more insights into the possible combined therapy correlated with inhibition of NFκB activation and cell death induction, experiments have been performed with PDTC, a compound that stimulates the cytotoxic impact of TNF-a on human Jurkat T-cells by suppressing the release of IκB from latent NFκB, due to a combination of antioxidant and heavy

metal chelating properties[39]. Our data show that PDTC has increased the cytotoxicity of C2-ceramdie on HT29 cells, which supports the hypothesis that the intracellular signaling pathway triggered by C2-ceramide is NFκB-dependent and prevents cell death. It has been demonstrated that NF-κB activation can inhibit caspase activity through the transcription of the c-iap gene[40]. Activation of NFκB by ceramide could explain why caspase activation is limited during ceramide-induced apoptosis.

However, some scientists hold that certain neuron cells result in apoptosis because of the expression of pre-apoptotic genes induced by activation of NFκB. Clemens *et al.*[41] have demonstrated that sustained activation of NFκB leads to accumulation of apoptosis in hippocampus nerve cells after 7 h of mouse transient cerebral ischemia. Shou *et al.*[42] have also reported that activation of NFκB could induce expression of pre-apoptotic proteins, Bcl-Xs and Bax, but has no effect on anti-apoptotic proteins, Bcl-2 or Bcl-xL, resulting in apoptosis. The modulation of activation of NFκB is complex. Further study is needed.

In summary, ceramide-induced apoptotic cell death involves caspase-dependent and -independent signaling pathway. Caspase-independent cell death occurring in the relatively early stage, which is mediated by p38 MAP kinase, can progress into a stage involving caspase-dependent and -independent mechanisms, as well as cell signaling of MAPKs and NFκB.

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