



Apoptosis of human colon carcinoma HT-29 cells induced by ceramide

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Abstract

AIM: To investigate the effect of exogenous ceramide-induced apoptosis on human colon carcinoma HT-29 cells.

METHODS: Light microscope, transmission electron microscope and fluorescence microscope were used to observe the morphology change of apoptosis in HT-29 cells. Agarose gel electrophoresis was performed to detect the DNA fragment. Mitochondrial function was detected by MTT assay. mRNA expression of Bcl-2 family gene members was determined by reverse transcription polymerase chain reaction (RT-PCR) assay.

RESULTS: After C₂-ceramide treatment, typical characteristics of apoptosis, such as nuclear chromatin breakage, apoptotic body and DNA ladder, could be observed. After exposure to 50 μ mol/L C₂-ceramide for 12 and 24 h, cell apoptosis was 64.1% and 81.3% respectively, which had a time-and dose-effect relationship. Mitochondrial function started to decrease from 6 h after exposure to ceramide. Meanwhile, ceramide up-regulated or down-regulated the mRNA expression of Bcl-2 family gene members.

CONCLUSION: Ceramide induces apoptosis of human colon carcinoma HT-29 cells by affecting the expression of Bcl-2 family gene members and impacting the mitochondrial function.

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Key words: Ceramide; Apoptosis; Human colon carcinoma cells; Bcl-2 family gene member; Mitochondrial function

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INTRODUCTION

Ceramide (N-acetyl-sphingosine) is a fundamental unit of sphingomyelin which is a moiety of cytoplasmic membrane. Ceramide, also an important bioactive compound *in vivo*, can conduct specific signal transduction from cell surface receptor and environmental stress to nuclei, and then provoke multiple cytobiologic effects^[1]. Meanwhile, ceramide is a common second messenger molecule participating in sphingomyelin cycle, apoptosis and differentiation of many cell types^[2,3]. Apoptosis can be induced by surrounding stimulating factors, such as tumor necrosis factor α (TNF- α), endotoxin, FAS ligand, radiation, chemotherapeutics and heat shock, and is associated with ceramide. Recent studies indicate that ceramide has a close relationship with genesis and progression of digestive tract tumor. But little information about Bcl-2 family gene member and mitochondrial function is available concerning the effect of ceramide-induced apoptosis in colon carcinoma which is one of the most aggressive forms of cancer. Even ceramide could induce apoptosis of HT-29 cells. This study was to determine how ceramide induces apoptosis of human colon carcinoma cells and to discuss its mechanism.

MATERIALS AND METHODS

Materials

C₂-ceramide (N-acetyl-D-sphingosine) was purchased from Sigma, dissolved to 5 mmol/L in DMSO as a stock solution. Hoechst 33 258 and MTT were purchased from Sigma, dissolved in PBS solution. The two solutions were preserved at 4°C from light. Apoptosis DNA extract kit was from Shanghai Huashun Biotechnology Company. RPMI-1640 media, DMSO and fetal bovine serum (FBS) were bought from GIBCO. Trypsin and EDTA were obtained from Amresco.

Cell culture

HT-29 cells were provided by the Institute of Tumor Prevention and Cure in Beijing, China. Cells were cultured in RPMI-1640 media supplemented with 10%

Table 1 Primer sequences of Bcl-2 family gene members

Gene	Primer sequence	Size (bp)	Temperature (°C)
Bax	Sense: 5'-CCAGCTGCCCTTGGACTGT-3'	135	61
	Antisense: 5'-ACCCCTCAAGACCACTCTT-3'		
Bcl-xl	Sense: 5'-GTAAACTGGGGTCGCATTGT-3'	146	60
	Antisense: 5'-TGGATCCAAGGCTCTAGGTG-3'		
Bcl-2	Sense: 5'-GGCAATGTGACTTTTCCAA-3'	137	55
	Antisense: 5'-GGCTGATATTCTGCAACACTG-3'		
Bad	Sense: 5'-AGGGCTGACCCAGATTCC-3'	178	60
	Antisense: 5'-GTGACGCAACGGTTAAACCT-3'		
Bid	Sense: 5'-GCTTCCAGTGTAGACGGAGC-3'	116	60
	Antisense: 5'-GTGCAGATTCATGTGTGGATG-3'		
β-actin	Sense: 5'-GTGGACATCCGCAAGAC-3'	303	58
	Antisense: 5'-TCAACGCAATGTGGAAG-3'		

FBS and 2 mmol/L glutamine. Antibiotics added to the media were 100 U/mL penicillin and 100 U/mL streptomycin. Cells maintained in a humidified atmosphere of 95 mL/L air and 50 mL/L CO₂ at 37°C were then passed at pre-confluent densities in a solution containing 0.25% trypsin and 0.02% EDTA. Exponentially growing cells were used throughout all experiments.

Light microscopy

HT-29 cells were treated with C₂-ceramide at final concentrations of 12.5, 25 and 50 μmol/L for 24 and 48 h respectively. DMSO served as negative control. During the procedure, cell morphology was observed under light microscope for different time.

Transmission electron microscopy

Cells were collected and fixed with 4% glutaraldehyde in phosphate buffer overnight at 4°C. After post-fixation with 1% OsO₄ in cacodylate buffer for 1 h at 4°C, the pellet was dehydrated in graded ethanol solutions and embedded in Epon. Ultrathin sections of pellet were counterstained with uranyl acetate and lead citrate and observed under transmission electron microscope.

Fluorescence microscopy

Cells (4 × 10⁴) were treated as above, suspended with FBS and incubated with Hoechst 33 258 for 30 min at 37°C from light. Then the cells were observed under fluorescence microscope. Three hundred cells were counted and apoptotic cell rate was calculated.

Detection of DNA fragmentation

Cells (2 × 10⁶) were treated as above and collected. Fragmented DNA was extracted according to the manufacturer's instructions of apoptosis DNA extract kit and underwent electrophoresis on 2 g/L agarose gel containing 0.5 g/L of ethidium bromide and visualized by UV transillumination.

Detection of mitochondrial function (MTT assay)

Cells (1.5 × 10⁴) were cultured in 96-well plates and treated with C₂-ceramide for 1, 3, 6, 9, 12 and 24 h as above. Then MTT and DMSO were added by turns. Absorbance was

Table 2 Apoptosis rate of HT-29 cells induced by C₂-ceramide (mean ± SD, %)

C ₂ -ceramide	Cell count (n)	12 h	24 h
0 μmol/L	300	5.33 ± 1.53	8.67 ± 2.52
12.5 μmol/L	300	14.7 ± 2.52 ^a	28.0 ± 9.85 ^a
25 μmol/L	300	33.3 ± 8.37 ^a	43.3 ± 12.3 ^a
50 μmol/L	300	64.7 ± 7.51 ^a	81.3 ± 9.07 ^a

^aP < 0.05 vs control.

determined with a multi-well plate reader at wavelength 570 nm.

RT-PCR

Cells (1 × 10⁶) were treated for 24 h as above. Total RNA was isolated from the treated cells with Trizol and reversely transcribed into cDNA with human specific primers for Bax, Bad, Bid, Bcl-2, Bcl-xl and β-actin. Sequences of primers are shown in Table 1. Briefly, 35 cycles of PCR amplification were performed at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s in a 25 μL reaction system. PCR products were confirmed by 1.5 g/L agarose gel electrophoresis and visualized by UV transillumination. mRNA expression of genes was assessed by correcting housekeeping gene β-actin, which served as an internal control.

Statistical analysis

All statistical analyses were performed with SPSS 10.0 statistical package for Microsoft Windows. Data were expressed as mean ± SD for all measurements. P < 0.05 was considered statistically significant.

RESULTS

C₂-ceramide induced morphology changes of apoptosis in HT-29 cells

With increasing concentration of C₂-ceramide and exposure time, HT-29 cells became round, atrophic and poorly adherent and floating cells increased under light microscope. Cells from control showed normal distribution and morphology in all cellular organelles except for a few necrotic cells under transmission electron microscope. The main ultra-microstructural changes seen in all treated groups were chromatin aggregation, mitochondrial denaturation and apoptotic body, as well as cytoplasmic compartments, swelling and disappearance of mitochondrial cristae, etc (Figure 1). The necrotic changes were more pronounced in cells treated with 50 μmol/L C₂-ceramide.

Apoptotic cells determined by Hoechst 33 258 assay increased in a time- and dose-dependent manner after treatment with C₂-ceramide (Table 2). Normal cellular chromatin did not change and uniformly spread over the whole nuclei displaying diffusion uniformity fluorescence. However, apoptotic chromatin was identifiable by its scattered drop-like structure locating on the area of the original nuclei which displayed high brightness lump or punctiform fluorescence. The total size of apoptotic nuclei

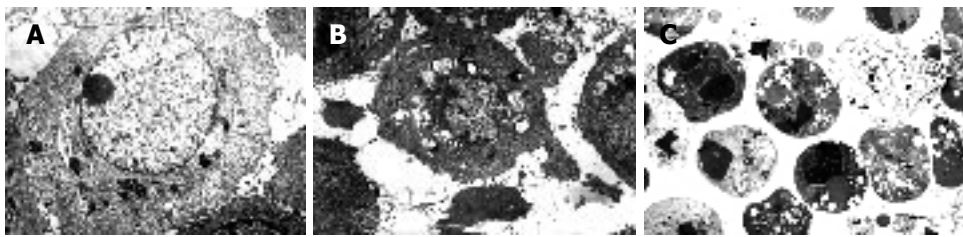


Figure 1 Morphology change of apoptosis in HT-29 cells induced by C₂-ceramide at different concentrations for different time under transmission electron microscope. **A:** 0 $\mu\text{mol/L}$, 24 h, $\times 5000$; **B:** 50 $\mu\text{mol/L}$, 24 h, $\times 4000$; **C:** 50 $\mu\text{mol/L}$, 48 h, $\times 3000$.

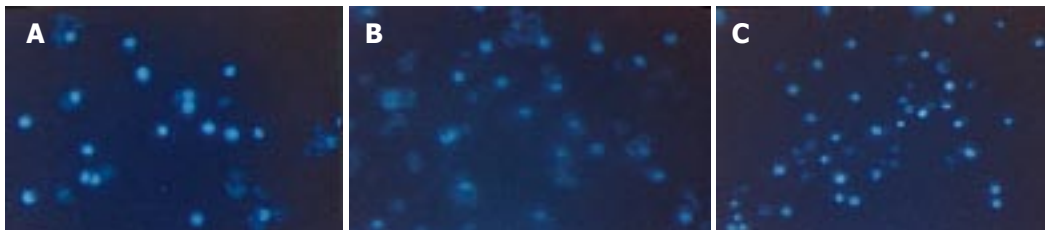


Figure 2 Morphology change of apoptosis in HT-29 cells induced by C₂-ceramide at different concentrations for different time under fluorescence microscope. **A:** 0 $\mu\text{mol/L}$, 24 h, $\times 100$; **B:** 50 $\mu\text{mol/L}$, 12 h, $\times 100$; **C:** 50 $\mu\text{mol/L}$, 24 h, $\times 100$.

Table 3 C₂-ceramide damaged mitochondria function of HT-29 cells (mean \pm SD)

C ₂ -ceramide ($\mu\text{mol/L}$)	1 h	3 h	6 h	9 h	12 h	24 h
0	0.505 \pm 0.061	0.596 \pm 0.050	0.528 \pm 0.083	1.088 \pm 0.134	1.371 \pm 0.066	1.770 \pm 0.161
12.5	0.478 \pm 0.044	0.609 \pm 0.048	0.502 \pm 0.029	0.833 \pm 0.149 ^a	1.036 \pm 0.122	1.560 \pm 0.220
25	0.487 \pm 0.051	0.568 \pm 0.048	0.404 \pm 0.033 ^a	0.646 \pm 0.158 ^a	0.760 \pm 0.086 ^a	1.484 \pm 0.346 ^a
50	0.490 \pm 0.054	0.541 \pm 0.049	0.345 \pm 0.050 ^a	0.456 \pm 0.071 ^a	0.708 \pm 0.073 ^a	1.342 \pm 0.061 ^a

^a $P < 0.05$ vs control.

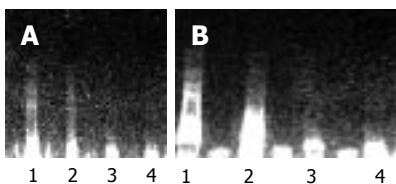


Figure 3 DNA fragmentation induced by C₂-ceramide in HT-29 cells for 12 h (**A**) and 24 h (**B**). 1: 50 $\mu\text{mol/L}$; 2: 25 $\mu\text{mol/L}$; 3: 12.5 $\mu\text{mol/L}$; 4: 0 $\mu\text{mol/L}$.

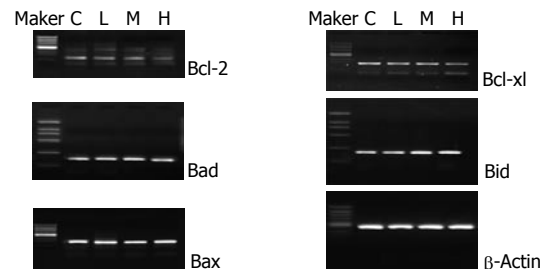


Figure 4 Expression of apoptosis-related genes in HT-29 cells induced by C₂-ceramide for 24 h (C: 0 $\mu\text{mol/L}$; L: 12.5 $\mu\text{mol/L}$; M: 25 $\mu\text{mol/L}$; H: 50 $\mu\text{mol/L}$).

appeared smaller and more shrunken than the intact cells (Figure 2).

C₂-ceramide induced DNA fragmentation of HT-29 cells

DNA ladder was seen through DNA agarose gel electrophoresis, especially pronounced in the cells treated with 50 and 25 $\mu\text{mol/L}$ C₂-ceramide for 12 and 24 h. Cells treated with 12.5 $\mu\text{mol/L}$ C₂-ceramide and DMSO kept their integrity, having no ladder appearance (Figure 3).

C₂-ceramide damaged mitochondrial function of HT-29 cells

The absorbance value increased gradually with increased concentration of C₂-ceramide and prolongation of exposure time. But the absorbance value after treatment with 50 and 25 $\mu\text{mol/L}$ C₂-ceramide was obviously lower than that in the control, showing statistical significance from 6 to 24 h, indicating that C₂-ceramide could decrease mitochondrial function (Table 3).

C₂-ceramide affected mRNA expression of apoptosis-related genes

After cells were treated with C₂-ceramide for 24 h, the mRNA expressions of Bax, Bad and Bid genes were up-regulated. However, the expressions of Bcl-2 and Bcl-xl were down-regulated (Figure 4). Moreover, the ratio of Bcl-2 to Bax in 50 $\mu\text{mol/L}$ C₂-ceramid group was less than 1.

DISCUSSION

Recently, the importance of ceramide in cell metabolism has been broadly investigated. The biological effect of ceramide on different cell lines is different. The role of ceramide-induced apoptosis has been confirmed^[4]. Recent studies indicate that ceramide is a common second

messenger molecule of apoptosis. Apoptosis induced by stimulating factors is mediated by ceramide from sphingomyelin circulation way^[5,6]. The effect of ceramide on apoptosis has been studied extensively in neoplastic cells but rarely in solid tumor cells. Ceramide is closely related with genesis and progression of digestive tract tumor. Decreased ceramide content increases the risk of developing digestive tract tumor since ceramide content in human colon carcinoma cells is lower than that in normal colon mucosa cells^[7].

To some extent, tumor chemotherapy is to induce apoptosis. In this experiment, human colon cancer cells were exposed to exogenous C₂-ceramide. Results indicated that C₂-ceramide could induce typical characteristics of apoptosis, such as nuclear chromatin break and apoptotic body as well as DNA ladder in a time-and dose-dependent manner. Succinate dehydrogenase (SDH) in the mitochondria is an index of cellular respiration and energy, which reflects mitochondrial function. MTT assay suggested that C₂-ceramide could decrease mitochondrial function. By studying Bcl-2 family gene members, we also found that C₂-ceramide could up-regulate or down-regulate the mRNA expression of these genes, suggesting that exogenous C₂-ceramide induces apoptosis of human colon carcinoma cells *in vitro* by affecting the expression of Bcl-2 family gene members and damaging the mitochondrial functions.

C₂-, C₆ and C₈-ceramides could induce apoptosis of cell lines, but dihydroxy-ceramide lacking C₄-C₅ trans-double bond located in basal framework of sphingolipid could not induce apoptosis^[8].

Studies also indicate that ceramide has many target sites inducing apoptosis, such as ceramide-activated protein kinase (CAPK)^[9], ceramide-activated protein phosphatase (CAPP)^[10], protein kinase C (PKC) family^[11], stress-activated protein kinase/c-JUN N-terminal kinase (SAPK/JNK)^[12] and caspase cascade reaction^[13]. There is a body of evidence that mitochondria are involved in apoptosis, for example, release of cytochrome C from mitochondria triggers apoptosis^[14-16].

In conclusion, mitochondria is the target of ceramide. The importance and mechanism of mitochondria in ceramide-induced apoptosis need to be further studied.

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