ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Mar；21 (3)：225 – 228
http：//www.chinaphar.com

Apoptosis induced by ceramide in hepatocellular carcinoma Bel7402 cells

ZHU Xiao-Feng[1]，ZHANG Xiao-Shi，LI Zhi-Ming，YAO Yu-Qi，XIE Bing-Fen，LIU Zong-Chao，ZENG Yi-Xin[1]
(*Department of Therapeutic Basis，Cancer Center，Sun Yat-sen University of Medical Sciences，Guangzhou 510060，China*)

KEY WORDS ceramides；apoptosis；cultured tumor cells；agarose gel electrophoresis；flow cytometry；fluorescence microscopy；Western blotting；hepatocellular carcinoma

ABSTRACT

AIM：To study the biological function of ceramide signaling in Bel7402 cells. METHODS：Inhibition of cell growth was assayed using MTT method. Morphologic assessment of apoptosis was performed with fluorescence microscope. DNA fragmentation was detected by electrophoresis and flow cytometry. The levels of protein p53，Bcl-2，and Bax were measured with Western blot. RESULTS：Bel7402 cells treated with C_2-ceramide underwent cell proliferation inhibition. IC_{50} value was 14.28 μmol·L^{-1}. After treatment of Bel7402 with ceramide，the morphologic changes including reduction in volume，nuclear chromatin condensation，fluorescence strength were observed. $SubG_1$ peaks were detected on flow cytometry (FCM). Agarose gel electrophoresis of DNA from cells treated with ceramide revealed "ladder" pattern. The Western blot assay from cell extracts showed that the levels of protein p53 were decreased after ceramide treatment. The levels of protein Bcl-2 were decreased also. But the levels of Bax protein showed no difference between untreated cells and treated cells. CONCLUSION：Ceramide induces apoptosis in Bel7402 cells，related to Bcl-2 down-regulation.

INTRODUCTION

Ceramide mediates differentiation，growth arrest，apoptosis，proliferation，cytokine biosynthesis and secretion，and a variety of other cellular functions[1]. Many agents including radiation，chemotherapeutic drugs，heat shock *etc*，can stimulate ceramide signaling[2]. Ceramide is thought to be involved in modulating ceramide-activated protein kinase (CAPK) and Ca^{2+}-calmodulin dependent protein kinase (CCDPK，formally called MAPK)，ceramide-activated protein phosphatase (CAPP)，and phospholipase A2 (PLA2) *etc*[3]. Rb phosphoration，SAPK/JNK pathway，and caspase can be activated. On the other hand，activation of PKC，Bcr-abl，Bcl-2 expression can inhibit ceramide signaling pathway[4-6]. The specific signaling pathway activated by ceramide depends on the cell type. The challenge for investigation in this field is to determine general mechanism through which ceramide can signal. Ceramide activation of the JNK cascade apoptosis is antagonized by another lipid second messenger sphingosine-1-phosphate，a kind of ceramide metabolism[7]. In this experiment，the effect of ceramide on hepatocellular carcinoma Bel7402 cells was studied.

MATERIALS AND METHODS

Drugs and reagents C_2-ceramide (N-acetylsphingosine) was initially dissolved in 100 % Me_2SO and stored at – 20 ℃. MTT was purchased from Janssen Chimica Co. RPMI-1640 medium，propidium iodide，C_2-ceramide，Me_2SO were purchased from Sigma Co. Anti-p53，Bcl-2，Bax antibody were purchased from Santa Cruz. Bel7402 cell line was provided by Cancer Institute，Sun Yat-sen University of Medical Sciences.

MTT assay Bel7402 cells (2000) were added to 96-well plates. Ceramide was diluted，then added to the wells for the desired final assay concentration. After 72-h exposure to ceramide，10 μL MTT (5 g/L) was added to every well and incubated for 4 h，then the liquid was transferred to the wells. Me_2SO 0.2 mL was added to every well. The absorbance was detected in the ELISA plate reader. Growth inhibitory rates were expressed as a

[1] Correspondence to Dr ZHU Xiao-Feng or Prof ZENG Yi-Xin.
Phn 86-20-8776-5368，ext 7333. Fax 86-20-8775-4506.
E-mail xfzhu@gzsums.edu.cn
Received 1999-05-18 Accepted 1999-08-25

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Mar；21 (3)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

percentage of absorbance detected in control wells that were treated with 0.1 % Me_2SO alone. Me_2SO controls were not different from cells in regular growth medium. IC_{50} values were determined using a Bliss Software.

Fluorescence staining Cells were collected by centrifugation (2000 × *g* for 5 min), resuspended in 20 μL of PBS, and stained in 10 μL of Hoechst 33342 solution (100 μmol·L^{-1}) in PBS. Cells were incubated for 30 min before examination with an Olympus photomicroscope with an epifluorescence attachment.

Flow cytometry C_2-ceramide or Me_2SO (0.1 %) was added to Bel7402 cells in mid-logarithmic phase (1×10^9 cells/L). After 24 h, 1×10^6 cells were collected, pelleted, washed with PBS, and resuspended in a final volume of 200 μL of ice-cold PBS. 1 mL of 70 % (vol/vol) ethanol in PBS was added to the resuspended cells with vigorous mixing. Fixed cells were incubated in the dark at 4 ℃ overnight. Cells were rehydrated in 300 μL of PBS for 25 min and then stained for 5 min with propidium iodide (final concentration, 16 μmol·L^{-1}) before flow analysis. Cells (10 000) were assessed with respect to their red fluorescence profile (575 ± 26 nm) using 488 nm excitation at 150 mW from a Coherent Enterprise Laser of a FACS vantage instrument (Becton Dickinson). Resulting DNA histograms were acquired using LYSIS Ⅱ software.

Internucleosomal DNA damage The integrity of DNA was assessed by agarose gel electrophoresis. Bel7402 (1×10^6) cells were centrifuged at 3000 × *g* for 3 min. Cells were washed once with PBS, and cell pellet was solubilized in 100 μL lysis buffer (Tris-HCl 50 mmol·L^{-1}, pH 8.0, tetraacetic acid 10 mmol·L^{-1}, 0.4 % SDS, 0.5 g/L proteinase K). Pellets were incubated at 50 ℃ for 8 h, then RNaseA 0.5 g/L 10 μL were added. The samples were incubated at 50 ℃ for an additional 1 h and heated to 70 ℃, then 100 μL phenol∶chloroform∶isopropranol (25 ∶ 24 ∶ 1) was added. After centrifugation, supernatants were transferred to new tubes, and two fold volume ethanol (ice cold) was added. After centrifugation, the pellets were loaded on 1.8 % agarose gel for electrophoresis, stained with ethidium bromide, and photographed with UV illumination.

Western blot analysis of p53, Bcl-2, and Bax Lysates were prepared from 1×10^6 cells by dissolving cell pellets in SDS-PAGE sample buffer (Tris-HCl 0.125 mol·L^{-1}, pH 6.8, 2 % SDS, 10 % glycerol, DTT 0.2 mol·L^{-1}). Lysates were heated to 100 ℃ for 5 min, and 100 μg of proteins were loaded in 15 % SDS-PAGE gel. Resolved proteins were electrophoretically transferred to nitrocellulose and incubated sequentially with anti-human p53, Bcl-2, Bax monoclonal antibody (Santa Cruz) and horseradish peroxidase-conjugated goat anti-mouse IgG (or anti-rabbit-IgG, Amershan Life Sciences). After washing, the bound antibody complex was detected using an ECL chemiluminescence reagent and XAR film (Kodak) as described by the manufacturers (Amershan).

RESULTS

Inhibition of growth by ceramide in Bel7402 Under concentration of 6.25, 12.5, 25, 50 μmol·L^{-1}, growth inhibitory rates of C_2-ceramide on Bel7402 cells were 29 % ± 2 %, 32 % ± 6 %, 71 % ± 4 %, 92 % ± 5 %. IC_{50} value was 14.28 (12.40 – 16.46) μmol·L^{-1}. In light microscopy, the control cells were attached to wells and touched each other. But the cells treated with ceramide underwent growth arrest and detached from each other.

Fluorescent staining Hoechst 33342 staining of cells after 48-h drug exposure revealed extensive nuclear chromatin condensation, reduction in cell volume and fluorescence strength, as well as the appearance of apoptotic bodies in Bel7402 cells treated with ceramide 25 μmol·L^{-1}. In contrast, 0.1 % Me_2SO-treated Bel7402 cells and nuclei remained morphologically indistinguishable from control cells (Fig 1).

Flow cytometry of ceramide-treated cells Ceramide (12.5, 25, 50 μmol·L^{-1}) and Me_2SO (0.1 %)-treated Bel7402 cells were stained for 24 h with propidium iodide and cell cycle distribution was analyzed by flow cytometry. The profiles of the DNA histograms were strickingly different from untreated Bel7402 cells. Bel7402 cells after treatment with ceramide exhibited a $SubG_1$ peak and the apoptotic index was 11.9 %, 18.5 %, 40.8 %, respectively.

Internucleosomal DNA damage in ceramide-treated Bel7402 cells DNA was prepared from 1×10^6 – 2×10^6 Bel7402 cells that had been treated with ceramide 50 μmol·L^{-1}. The integrity of the DNA was assessed by agarose gel electrophoresis (Fig 3). Internucleosomal DNA damage in Bel7402 cells was readily detected at 24 h after treatment with ceramide 50 μmol·L^{-1}. By 48 h DNA ladders were clearly observed on agarose gel.

Effect of ceramide on protein p53, Bcl-2,

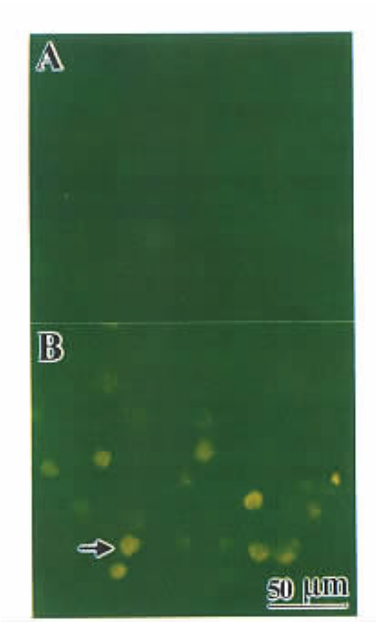

Fig 1. Fluorescent micrographs of Bel7402 cells treated with 0.1 % Me_2SO (A) or ceramide 25 μmol·L^{-1} (B) for 48 h (×200, Hoechst 33342 staining).

Fig 2. Flow cytometric analyses of Bel7402 cells treated with 0.1 % Me_2SO (A) or ceramide 50 μmol·L^{-1} (B) for 24 h.

Bax levels in Bel7402 cells Western blot analysis showed that the levels of protein p53 were decreased (Fig 4). The levels of protein Bcl-2 were decreased also (Fig 5). But the levels of protein Bax showed no change between untreated and treated cells (Fig 6).

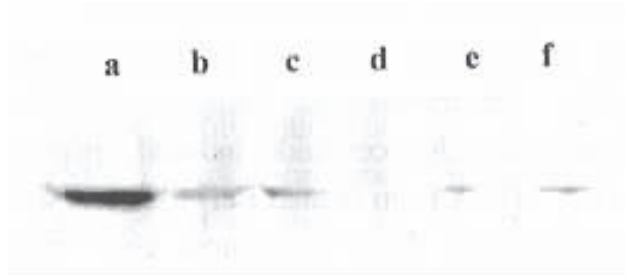

Fig 3. Internucleosomal DNA fragmentation in Bel7402 cells treated with 0.1 % Me_2SO (A), ceramide 50 μmol·L^{-1} for 12 h (B); for 24 h (C); or for 48 h (D).

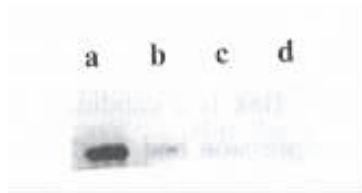

Fig 4. The changes in levels of protein p53 in ceramide-treated Bel7402 cells. a) 0.1 % Me_2SO; b) ceramide 50 μmol·L^{-1} treatment for 3 h; c) 6 h; d) 12 h; e) 24 h; f) 48 h.

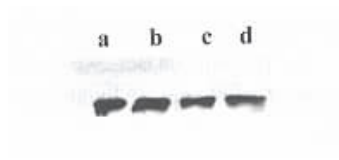

Fig 5. The changes in protein Bcl-2 levels in ceramide-treated Bel7402 cells. a) 0.1 % Me_2SO; b) ceramide 50 μmol·L^{-1} treatment for 6 h; c) 12 h; d) 24 h.

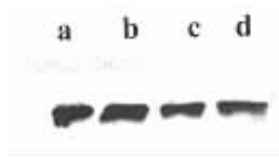

Fig 6. The changes in protein Bax levels by ceramide treatment in Bel7402 cells. a) 0.1 % Me_2SO; b) 6 h; c) 12 h; d) 24 h.

DISCUSSION

Ceramide is a kind of inhibitory second messenger. Ceramide signaling pathway is a conserved signal system.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Mar；21 (3)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Like cAMP and IP_3 pathway, most of mammalian cells can transmit ceramide signaling. The sphingomyelin is preferentially concentrated in the plasma membrane of mammalian cells. Sphingomyelin hydrolysis occurs via the action of sphingomyelin specific forms of phospholipase C, termed sphingomyelinases. Ceramide, generated by this reaction, acts as a second messenger[8] and initiates cell type specific signaling. In most instances, analogs of ceramide but not other lipid second messengers mimic the induction of the death response. Numerous reports provide evidence for a causal role for JNK activation in induction of apoptosis, while an equal number of studies suggest that JNK activation is either anti-apoptotic or non-apoptotic. Cell-type specificity may be one reason that consensus has not been reached for either a strictly pro- or anti-apoptotic role for JNK signaling[9].

In our experiment, ceramide inhibited growth of Bel7402 cells. The morphologic changes and DNA fragmentation suggested that ceramide induced apoptosis of Bel7402 cells. DNA from treated Bel7402 cells with ceramide showed typical ladder on agarose gel. p53 is a kind of tumor suppressor gene. It participates in apoptosis induced by many stimuli. When ceramide induced apoptosis in Bel7402, protein p53 level showed a decreased tendency. Induction of apoptosis by ceramide in Bel7402 can be through p53-independent pathway. Bcl-2 is a candidate in anti-apoptotic pathway. Our experiment showed that protein Bcl-2 was downregulated in ceramide treatment. Bax is a candidate in apoptotic pathway. But Bax expression had no difference between ceramide-treated cells and untreated cells. Bax can not be related to apoptosis induction by ceramide in Bel7402 cells. Bcl-2/Bax ratio is important to apoptosis induced by several agents. The decline of this ratio contributes to apoptosis induction. In this experiment, the levels of protein Bcl-2 decreased, but the levels of protein Bax did not change. So the ratio of Bcl-2/Bax decreased. This may be contributing to apoptosis induction by ceramide in Bel7402 cells.

REFERENCES

1 Hannun YA. Functions of ceramide in coordinating cellular responses to stress. Science 1996；274：1855－9.
2 Haimovitz-Friedman A, Kolesnick RN, Fuks Z. Ceramide signaling in apoptosis. Br Med Bull 1997；53：539－53.
3 Ballou LR, Laulederkind SJ, Rosloniec EF, Raghow R. Ceramide signalling and the immune response. Biochim Biophys Acta 1996；1301：273－87.
4 Zhang J, Alter N, Reed J, Borner C, Obeid LM, Hannum YA. Bcl-2 interrupts the ceramide-mediated pathway of cell death. Proc Natl Acad Sci USA 1996；93：5325－8.
5 Maguer-Satta V, Burl S, Liu L, Damen J, Chahine H, Krystal G, *et al*. BCR-ABL accelerates C_2-ceramide-induced apoptosis. Oncogene 1998；16：237－48.
6 Sawai H, Okazaki T, Takeda Y, Tahima M, Sawada H, Okuma M *et al*. Ceramide-induced translocation of protein kinase C-delta and epsilon to the cytosol. Implications in apoptosis. J Biol Chem 1997；272：2452－8.
7 Cuvillier O, Rosenthal DS, Smulson ME, Spiegel S. Sphingosine-1-phosphate inhibits activation of caspases that cleave poly (ADP-ribose) polymerase and lamins during Fas- and ceramide-mediated apoptosis in Jurkat T lymphocytes. J Biol Chem 1998；273：2910－6.
8 Gomez-MunozA. Modulation of cell signalling by ceramides. Biochim Biophys Acta 1998；1391：92－109.
9 Basu S, Kolesnick R. Stress signals for apoptosis：ceramide and c-*jun* kinase. Oncogene 1998；17：3277－85.

神经酰胺诱导肝肿瘤 Bel7402 细胞凋亡

朱孝峰[1]，张晓实，李志铭，姚毓奇，谢冰芬，刘宗潮，曾益新[1]（中山医科大学肿瘤防治中心治疗基础室，广州 510060，中国）

关键词 神经酰胺类；细胞凋亡；培养的肿瘤细胞；琼脂糖凝胶电泳；流式细胞术；荧光显微镜检查；蛋白质印迹；肝细胞癌

目的：了解神经酰胺信号转导在肝肿瘤细胞中的生物学意义. **方法：**MTT 法、荧光染色、流式细胞术、琼脂糖凝胶电泳、Western blot. **结果：**神经酰胺对 Bel7402 细胞生长抑制的 IC_{50} 值为 14.28 $\mu mol \cdot L^{-1}$. 细胞核浓缩，荧光明显增强；DNA 呈现典型“梯型”变化；p53 蛋白表达呈下降趋势；Bcl-2 蛋白表达明显降低；Bax 蛋白表达无明显改变. **结论：**神经酰胺诱导肝肿瘤细胞凋亡，与 Bcl-2 表达下调有关.

（责任编辑 朱倩蓉）