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# Induction of Apoptosis by Sphingosine-1-Phosphate in Human Hepatoma Cells Is Associated with Enhanced Expression of bax Gene Product

Wen-Chun Hung*,[1] and Lea-Yea Chuang†

**School of Technology for Medical Sciences and †Department of Biochemistry, Kaohsiung Medical College, Kaohsiung 807, Taiwan, Republic of China*

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Sphingolipid metabolites are important regulators of cell growth and differentiation. Recent studies have suggested that sphingosine-1-phosphate, a biologically active sphingolipid metabolite, acts as a crucial messenger in apoptosis. In the present work, we examined the expression levels of the members of the bcl-2-related gene family to determine their roles in sphingosine-1-phosphate-induced apoptosis in human hepatoma cells. Our results indicate that sphinogosine-1-phosphate-induced apoptosis is associated with enhanced expression of Bax protein. Moreover, the regulation of bax gene expression by sphingosine-1-phosphate is independent of the p53 tumor suppressor. 

Sphingolipids, a major class of membrane lipids, have been shown to be involved in diverse cellular processes (1-3). Sphingosine-1-phosphate is a bioactive sphingolipid metabolite which affects different signal transduction pathways in various types of cells. This small molecule acts as a second messenger in cell proliferation induced by platelet-derived growth factor and fetal calf serum (4-5). It also plays a crucial role in controlling cell motility and tumor cell invasiveness (6). Furthermore, recent studies have suggested that sphingosine-1-phosphate may be one of the regulators of apoptosis caused by different stimuli. This sphingolipid metabolite has been reported to block the apoptotic cell death induced by ceramide and tumor necrosis factor-$\alpha$ (7). Conversely, sphingosine-1-phosphate may induce apoptotic cell death in some types of cells (8).

Apoptosis initiated by many external signals could be regulated by the members of the bcl-2 gene family (9,10). Within this gene family, some can suppress apoptosis while others stimulate it. Overexpression of bax or bcl-xs gene product caused apoptosis in cells (11,12). On the contrary, enhanced expression of bcl-2 or bcl-x gene product may protect cells from apoptosis induced by different stimuli (13,14).

The molecular mechanism of apoptosis triggered by sphingosine-1-phosphate (or other bioactive sphingolipid metabolites) in human cancer cells is not understood. In this study, we investigated the profile of protein expression of the members of bcl-2 gene family during apoptosis initiated by sphingosine-1-phosphate in Hep3B hepatoma cells to clarify their roles in sphingolipid-induced apoptosis.

## MATERIALS AND METHODS

*Cell culture.* Hep3B human hepatoma cells were maintained in DMEM/F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 $\mu$g/ml streptomycin and 100 IU/ml penicillin in a 5% $CO_2$ incubator at 37°C.

*Materials.* D-erythro-sphingosine-1-phosphate was obtained from the Calbiochem-Novabiochem International (San Diego,CA,USA) and was dissolved in methanol. Antibodies to Bcl-2, Bax, Bcl-x, and Bcl-xs were purchased from

[1] To whom correspondence should be addressed. Fax: 886-7-3113449.



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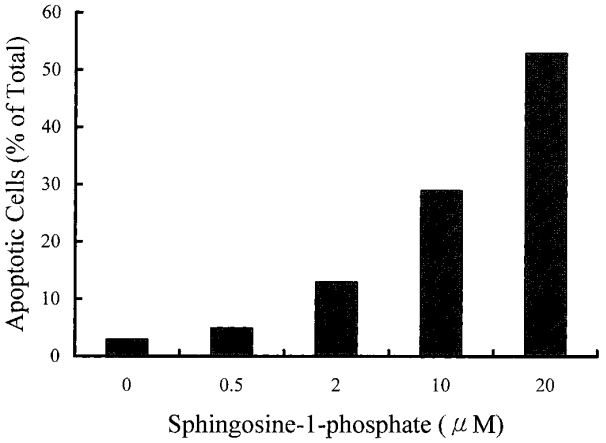


**FIG. 1.** Sphingosine-1-phosphate induces apoptosis in Hep3B hepatoma cells. Cells were treated with different concentrations of sphingosine-1-phosphate for 48 h. Apoptotic cells which permitted trypan blue uptake were counted and expressed as a percentage of the total cell number.

Oncogene Research Products (Cambridge,MA,USA). All these antibodies are peptide-specific rabbit polyclonal antibodies. The ECL chemiluminescence system was purchased from Amersham (Arlington Heights,IL,USA).

*Cell viability assays.* Cells were seeded at a density of $2\times10^5$ cells/well in 6-well plates in 10% FCS medium. After 24 h, cells were washed with phosphate-buffered saline (PBS) and maintained in 0.5% FCS medium containing different concentrations of sphingosine-1-phosphate for another 48 h. After incubation, the detached cells were pelleted by centrifugation and the attached cells remained in the flasks were collected with cell scrapers. Apoptotic cells which permitted trypan blue uptake were counted and expressed as a percentage of the total cell number.

*Extraction of DNA and agarose gel electrophoresis.* Cells were cultured to confluence in 75 $cm^2$ culture flasks in 10% FCS medium. Cells were washed twice with PBS and incubated with 20 $\mu$M sphingosine-1-phosphate in 0.5% FCS medium for different time intervals. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1% SDS and 500 $\mu$g/ml proteinase K). Analysis of DNA fragmentation was performed as described previously (15).

*Western blot analysis.* Cells were treated with 20 $\mu$M sphingosine-1-phosphate for different time intervals. Cells were washed with PBS and harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 500 $\mu$M sodium orthovanadate, 1 mg/ml aprotinin, 1 mM phenylmethyl-sulfonyl fluoride, 10 $\mu$g/ml leupeptin and 10 $\mu$g/ml pepstain A). Protein concentrations were determined with a DC protein assay kit (Bio-Rad, Hercules, CA). The cell lysates (50 $\mu$g protein/sample) were subjected to a 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The expressions of Bcl-2, Bax, Bcl-x and Bcl-xs proteins were investigated by immunoblotting as described previously (16). The concentration of primary antibodies used to probe the nitrocellulose blots was 3 $\mu$g/ml.

## RESULTS AND DISCUSSION

As shown in Fig. 1, sphingosine-1-phosphate induced apoptosis in Hep3B cells in a dose-dependent manner. Treatment with 20 $\mu$M sphingosine-1-phosphate for 48 h caused apoptotic cell death in 53% in these cells. Next, we examined the time course of activation of apoptosis induced by sphingosine-1-phosphate. Hep3B cells incubated with 20 $\mu$M sphingosine-1-phosphate were harvested at different time intervals and cell viability was examined by trypan blue exclusion test. After counting, cells were then lysed and DNA samples were prepared as described in ''Materials and Methods''. DNA was separated by electrophoresis in agarose gel to investigate the characteristic pattern of internucleosomal fragmentation in apoptosis. Our results showed that apoptotic cell death of Hep3B cells induced by sphingosine-1-phosphate was evidenced after continuous treatment with this sphingolipid metabolite for 12 h (Fig. 2). Fig. 3 indicated that fragmentation of nuclear DNA was also found at similar time point (12 h after incubation). Our data is somewhat different from that reported by Sweeney EA et al. (17). In their study, treatment with

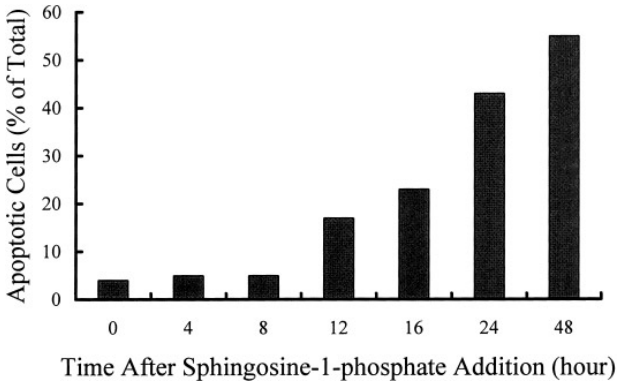


**FIG. 2.** Time-dependent activation of apoptotic cell death by sphingosine-1-phosphate in Hep3B human hepatoma cells. Cells were treated with 20 $\mu$M sphingosine-1-phosphate for different time intervals. Viable cells were determined by trypan blue exclusion test.

sphingosine and its methylated derivative N,N-dimethylsphingosine (20 $\mu$M) for 6 h caused apoptosis in most of the hematopoietic and carcinoma cell lines tested. One of the explanations of this discrepancy is that the apoptotic pathways activated by different sphingolipid metabolites may different. Our previous study also indicated that multiple apoptotic pathways coexist in Hep3B cells (15). Another possibility is that these sphingolipid metabolites must convert to other lipid mediators to inducing apoptosis. Indeed, Cuvillier O. et al. have noted recently that high concentrations of sphingosine-1-phosphate may trigger apoptosis in some cell types by conversion to sphingosine and re-acylation to ceramide, an important mediator in apoptosis caused by tumor necrosis factor-$\alpha$ and Fas ligand (7).

To date, the roles of the Bcl-2 related proteins in sphingolipid-induced apoptosis are totally unknown. We investigated the expression levels of Bcl-2, Bax, Bcl-x and Bcl-xs during apoptosis initiated by sphingosine-1-phosphate to examine whether changes in the expression of these proteins modulate apoptosis. We found that the expressions of Bcl-2, Bcl-x and Bcl-

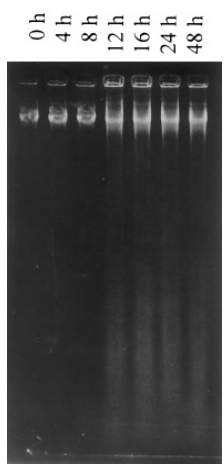


**FIG. 3.** Sphingosine-1-phosphate induces DNA fragmentation in Hep3B cells. Cells were incubated with 20 $\mu$M sphingosine-1-phosphate for different time intervals. DNA samples were extracted and prepared as described under Materials and Methods. Nuclear DNA was subjected to a 1.5% agarose gel electrophoresis.

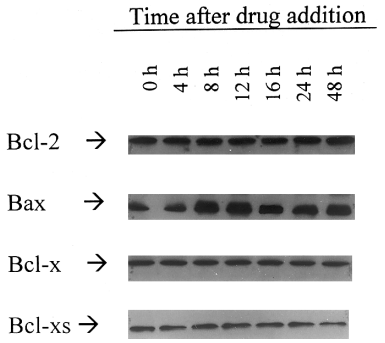


**FIG. 4.** Analysis of the expressions of the Bcl-2-related proteins during apoptosis triggered by sphingosine-1-phosphate in Hep3B cells. Cells were treated with 20 $\mu$M sphingosine-1-phosphate for different time intervals and harvested in lysis buffer. The cell lysates (50 $\mu$g/sample) were subjected to SDS-PAGE, transferred to nitrocellulose paper, and probed with peptide-specific rabbit polyclonal antibodies.

xs were not regulated by sphingosine-1-phosphate (Fig. 4). On the contrary, the expression of Bax protein was strongly enhanced by this sphingolipid metabolite.

Bax is a 21 kDa protein with extensive amino acid homology with Bcl-2 (18,19). This protein has been shown to form heterodimers with Bcl-2 and the ratio of Bcl-2/Bax determines the survival or death following various apoptotic stimuli (20,21). In addition, recent data has indicated that tumor suppressor p53 is a direct transcriptional activator of the human bax gene and Bax is involved in a p53-regulated pathway for induction of apoptosis (22). Fig. 4 showed that the expression of Bax protein is enhanced gradually after continuous incubation of sphingosine-1-phosphate for 4 h. Synthesis of Bax protein was peaked at a time point (8-10 h) just prior to inducing apoptosis by this sphingolipid metabolite. Thus, we suggest that sphingosine-1-phosphate may trigger apoptotic cell death in Hep3B cells by up-regulating the expression of Bax protein.

The regulation of bax expression by sphingosine-1-phosphate is unclear at present time. Although the human bax gene promoter region contains four motifs with homology to consensus p53-binding sites, it has been reported that only wild type p53 can stimulate the expression of bax gene. Since the p53 gene was found to be partially deleted in Hep3B cells and the transcripts of the p53 gene were undetectable in these cells (23), it is concluded that sphingosine-1-phosphate triggers bax gene expression in a p53-independent pathway. Another important motif located within the 5′-untranslated region of the bax gene is the CACGTG motif (22). This hexameric DNA sequence has been shown to represent a potential binding site for Myc and its homologs (including Mad, Mxi-1 and Max) (24-26). Further work must be taken to elucidate the role of Myc in sphingosine-1-phosphate-induced apoptosis. Additionally, it has been shown that tumor cells are more susceptible to apoptosis induced by sphingolipid metabolites than normal cells (17). Thus, study of the apoptotic pathways triggered by these sphingolipid metabolites is a potentially useful approach to anti-tumor therapy.

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