Activation of caspase-3-like proteases in apoptosis induced by sphingosine and other long-chain bases in Hep3B hepatoma cells

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Sphingosine and other long-chain bases (including sphinganine, dimethylsphingosine and stearylamine), but not octylamine (a short-chain analogue of sphinganine), induced apoptosis in Hep3B hepatoma cells. Because both D- and L-erythrospingosine and stearylamine exert potent apoptotic effects on Hep3B cells, it is possible that these long-chain bases may activate apoptosis by inhibiting protein kinase C (PKC) activity. However, pretreatment with the PKC activator PMA could not rescue cells from apoptosis triggered by long-chain bases. Therefore the involvement of PKC in this apoptotic process requires further characterization. We also investigated whether these long-chain bases might be metabolized into ceramide in order to elicit their apoptotic action. We found that long-chain bases acted independently of ceramide in the induction of apoptosis, since addition of fumonisins B1, a fungal agent which effectively inhibits ceramide synthesis from sphingosine, did not protect against apoptosis. Additionally, we found that sphingosine-

induced apoptosis was accompanied by activation of caspases. The functional role of caspases in this apoptotic process was examined by using specific caspase inhibitors. The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone, which exhibits a broad specificity for caspase-family proteases, effectively blocked sphingosine-induced apoptosis. Furthermore, our results indicate that caspase-3-like proteases, but not caspase-1, are activated during apoptosis triggered by sphingosine. Enhancement of caspase-3-like activity and cleavage of poly(ADP-ribose) polymerase, an *in vivo* substrate for caspase-3, was clearly demonstrated in sphingosine-treated Hep3B cells. Considered together, these results suggest that caspase-3-like proteases participate in apoptotic cell death induced by sphingosine.

Key words: caspase inhibitors, fumonisins B1, poly(ADP-ribose) polymerase, protein kinase C.

INTRODUCTION

Apoptosis, a specific type of cell death, is involved in the regulation of cell number in multicellular organisms, and in the pathogenesis of various diseases, including tumour progression, neurodegenerative disorders and viral infection [1,2]. Morphological changes observed in cells undergoing apoptosis include cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation [3]. The mechanisms of activation of apoptosis in different physiological or pathological conditions have been studied intensively. Recent studies have suggested that the members of the *bcl-2* gene family play an important role in the regulation of apoptosis [4]. The *bcl-2* gene was first identified at the chromosome break-point of t(14;18)-bearing B-cell lymphomas [where t(14;18) denotes a reciprocal chromosomal translocation between gene loci on chromosomes 14 and 18] [5,6]. It is now clear that some members of this family (such as Bcl-2, Bcl-Xl, Bcl-w, Bfl-1, Bcl-1, Bcl-1 and A1) can effectively inhibit apoptosis, while others (Bax, Bak, Bcl-Xs, Bad, Bid, Bik and Hrk) may conversely promote apoptosis [7]. Because cells that have undergone apoptosis can be recognized and removed by macrophages without the induction of inflammation, natural or synthetic agents that can specifically activate apoptotic pathways in cancer cells are expected to be valuable for the treatment of human cancer.

Sphingolipids are derivatives of a number of common backbones, termed long-chain (sphingoid) bases [8]. These lipids appear to be ubiquitous among eukaryotic organisms and have long been recognized as having roles in membrane structure. Recent discoveries have revealed that sphingolipids (ceramide, sphingosine, sphingosine 1-phosphate, etc.) are highly bioactive compounds and are involved in diverse cell processes, including cell-cell interaction, cell proliferation, differentiation and apoptosis [9,10]. Previously, we and others have reported that several sphingolipids can regulate the expression of members of the *bcl-2* gene family and trigger apoptosis in a variety of human cancer cell lines [11,12]. However, the downstream effectors activated by these sphingolipids in this apoptotic signalling pathway are still unknown.

A number of cysteine proteases have been implicated in the apoptotic process [13,14]. These proteases were initially identified from genetic analysis of programmed cell death in the nematode *Caenorhabditis elegans* [15]. An essential gene, *ced-3*, which participates in the induction of apoptosis, was subsequently found to have high protein-sequence identity with mammalian interleukin-1 β -converting enzyme (ICE) (now termed caspase-1) [16]. To date, 10 human homologues of ICE/CED-3 protease have been identified, and overexpression of these proteases triggers apoptosis in mammalian cells [17,18]. Phylogenetic analysis of caspases reveals that there are three subfamilies: an

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ICE, interleukin-1 β -converting enzyme; pNA, *p*-nitroanilide; PARP, poly(ADP-ribose) polymerase; PKC, protein kinase C; TNF α , tumour necrosis factor α ; DEVD-FMK, Asp-Glu-Val-Asp fluoromethyl ketone; YVAD-CMK, Tyr-Val-Ala-Asp chloromethyl ketone; ZVAD-FMK, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone.

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ICE subfamily, a CED-3/CPP32 subfamily and an ICH-1/Nedd2 subfamily (where ICH is defined as 'ICE and CED-3 homologue'). In the present work, we study the apoptotic signalling pathway activated by sphingosine and evaluate the functional role of caspases in this apoptotic pathway. Our results indicate that sphingosine induces caspase-3-like activity in Hep3B hepatoma cells.

MATERIALS AND METHODS

Reagents and cell culture

D-Erythrosphingosine, sphinganine, dimethylsphingosine, PMA and fumonisin B1 were obtained from Calbiochem–Novabiochem International (San Diego, CA, U.S.A.). L-Erythrosphingosine, stearylamine and octylamine were obtained from Sigma (St. Louis, MO, U.S.A.). The caspase inhibitors Tyr-Val-Ala-Asp chloromethyl ketone (YVAD-CMK), Asp-Glu-Val-Asp fluoromethyl ketone (DEVD-FMK) and benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (ZVAD-FMK), the monoclonal antibody against poly(ADP-ribose) polymerase (PARP) and the ApoAlert CPP32 assay kit were purchased from Clontech (Palo Alto, CA, U.S.A.). Hep3B cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10 % heat-inactivated fetal calf serum (FCS), 100 µg/ml streptomycin and 100 units/ml penicillin in a 5 % CO₂ incubator at 37 °C.

Cell viability assay and morphological evaluation

Cells were seeded at a density of 2×10^5 cells/well in six-well culture plates in DMEM/F12 medium containing 10 % (v/v) FCS. After 24 h, cells were washed with PBS and cultured in DMEM/F12 medium containing 0.5 % (v/v) FCS containing different caspase inhibitors for 2 h. Then sphingosine and other long-chain bases (20 µM) were added, and incubations were continued for another 6 or 24 h. Cells were then harvested and washed twice with PBS. Cells were fixed in 4 % paraformaldehyde in PBS for 15 min at room temperature and stained with Hoechst 33258 fluorescent dye (20 µg/ml) for 15 min at room temperature. A 5 µl portion of cells was placed on a glass slide and observed under a fluorescence microscope. For each sample, 500 stained nuclei were counted; apoptotic cells, which contained condensed chromatin fragments, were scored and expressed as a percentage of the total cell number.

Western blot analysis

Cells were treated with 20 µM sphingosine or 50 µg/ml etoposide for different time intervals and then harvested into an ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 % Triton X-100, 0.5 mM sodium orthovanadate, 1 mg/ml aprotinin, 1 mM PMSF, 2 µg/ml leupeptin and 2 µg/ml pepstatin A), and protein concentrations were determined with a DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). Cell lysates (50 µg of protein/sample) were then subjected to SDS/7.5 %-PAGE, and the expression and proteolysis of PARP was investigated as described previously [11]. The concentration of anti-PARP monoclonal antibody used to probe the nitrocellulose blots was 1 µg/ml.

Extraction of DNA and agarose-gel electrophoresis

Cells were incubated with 20 µM sphingosine in DMEM/F12 medium containing 0.5 % (v/v) FCS in the presence or absence of 50 µM ZVAD-FMK for 24 h. Cells were lysed in a lysis

buffer (20 mM Tris/HCl, pH 8.0, 0.1 mM EDTA, 1 % SDS and 0.5 mg/ml proteinase K). DNA samples were extracted and separated by agarose-gel electrophoresis, as described previously [19].

Caspase-3 activity assays

Cells were treated with 20 µM sphingosine or 50 µg/ml etoposide for 12 h in the absence or presence of 50 µM DEVD-FMK, and caspase-3 activity was determined by a colorimetric assay kit according to the manufacturer's procedure (Clontech). This assay is based on the spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) at 405 nm after cleavage from the labelled caspase-3 substrate DEVD-pNA.

RESULTS

Sphingosine and other long-chain bases induce apoptosis in Hep3B hepatoma cells

We have demonstrated previously that sphingosine 1-phosphate and sphingosine induce Hep3B cell death in a dose-dependent and time-dependent manner. These two sphingolipids at 20 µM triggered significant cell death in Hep3B cells, and the type of cell death induced by these agents was apoptosis, as confirmed by DNA fragmentation in the cells [11]. Whether this cytotoxic effect is specific to sphingosine is unknown, and we therefore investigated the effects of other long-chain bases on Hep3B cells. As shown in Figure 1, the apoptotic cells were evaluated by morphological changes; condensed chromatin fragments were seen under a fluorescence microscope after staining with the fluorochrome bis-benzimide (Hoechst 33258 dye) in drug-treated cells. We found that D-erythrosphingosine, L-erythrosphingosine, sphinganine and dimethylsphingosine had potent cytotoxic effects on Hep3B cells. More than 80 % of cells were dead after continuous incubation with 20 µM dimethylsphingosine for 24 h, while the percentage of apoptotic cells in D-erythrosphingosine-, L-erythrosphingosine- and sphinganine-treated cells was 55 %, 58 % and 51 % respectively (Table 1). Additionally, we found that a long-chain alkylamine, stearylamine, at 20 µM also triggered apoptosis (25 %) in Hep3B cells. In contrast, octylamine, a short-chain analogue of sphinganine, at this concen-

Figure 1 Induction of apoptosis by sphingosine in Hep3B cells

Cells were cultured in the absence or presence of 20 µM sphingosine in DMEM/F12 medium containing 0.5 % (v/v) FCS for 24 h. Cells were harvested by trypsinization, stained with Hoechst 33258 dye and observed under a fluorescence microscope. Apoptotic cells containing condensed chromatin and fragmented DNA were found in sphingosine-treated cell samples.

Table 1 Induction of apoptotic cell death in Hep3B hepatoma cells by sphingosine and other long-chain bases

Hep3B cells were treated with sphingosine or other long-chain bases (20 μ M) in DMEM/F12 medium containing 0.5% (v/v) FCS for 6 or 24 h. Cells were stained with Hoechst 33258 dye and observed under a fluorescence microscope. Apoptotic cells which contained condensed chromatin fragments were scored and expressed as a percentage of the total cell number measured. Results from three independent experiments are shown as means $\pm$ S.E.M.

Treatment	Apoptotic cells (% of total cell number)	
	6 h	24 h
Control	3 $\pm$ 1	2 $\pm$ 1
D-Erythrospingosine	12 $\pm$ 3	55 $\pm$ 7
L-Erythrospingosine	18 $\pm$ 3	58 $\pm$ 5
Sphinganine	14 $\pm$ 5	51 $\pm$ 5
Dimethylsphingosine	28 $\pm$ 7	83 $\pm$ 11
Stearylamine	8 $\pm$ 1	25 $\pm$ 3
Octylamine	5 $\pm$ 2	6 $\pm$ 3

Table 2 Effect of PMA on apoptosis induced by sphingosine and other long-chain bases

Cells were preincubated with PMA (10 nM) for 30 min and then treated with different long-chain bases (20 μ M) for another 6 or 24 h. Apoptotic cells were stained and scored as described in Table 1.

Treatment	Apoptotic cells (% of total cell number)	
	6 h	24 h
Control	2 $\pm$ 1	4 $\pm$ 1
PMA	4 $\pm$ 2	5 $\pm$ 3
D-Erythrospingosine	15 $\pm$ 6	53 $\pm$ 7
D-Erythrospingosine + PMA	12 $\pm$ 3	48 $\pm$ 3
L-Erythrospingosine	14 $\pm$ 3	53 $\pm$ 8
L-Erythrospingosine + PMA	16 $\pm$ 5	50 $\pm$ 5
Sphinganine	13 $\pm$ 2	49 $\pm$ 2
Sphinganine + PMA	15 $\pm$ 5	55 $\pm$ 6
Dimethylsphingosine	30 $\pm$ 5	83 $\pm$ 10
Dimethylsphingosine + PMA	27 $\pm$ 4	79 $\pm$ 5
Stearylamine	10 $\pm$ 2	23 $\pm$ 6
Stearylamine + PMA	12 $\pm$ 2	25 $\pm$ 8

tration did not have a significant apoptotic effect on Hep3B cells. Therefore, in agreement with other studies [20,21], long-chain bases potentially induce apoptosis in human cancer cells.

We attempted to elucidate the apoptotic signalling pathway activated by sphingolipids. Previous observations have indicated that sphingolipids may act via two important signalling molecules, protein kinase C (PKC) and ceramide, to elicit their biological effects. Therefore we examined whether PKC or ceramide is involved in this apoptotic process. We found that the PKC activator PMA could not antagonize the apoptotic effects of sphingosine or other long-chain bases in Hep3B cells after a 24 h incubation (Table 2). Since long-term incubation of cells with PMA leads to down-regulation of PKC and complicates interpretation of the results, we also checked the effect of PMA on cell viability at 6 h after sphingolipid addition. Again, no cytoprotective effect of PMA was found (Table 2). Although these data do not support the idea that sphingolipids may block PKC activity to induce apoptosis in Hep3B cells, we cannot

Table 3 Effect of fumonisin B1 on apoptosis induced by sphingosine and other long-chain bases

Cells were preincubated with fumonisin B1 (20 μ M) for 30 min and then treated with various long-chain bases (20 μ M) for another 6 or 24 h. Apoptotic cells were stained and scored as described in Table 1.

Treatment	Apoptotic cells (% of total cell number)	
	6 h	24 h
Control	2 $\pm$ 1	4 $\pm$ 1
Fumonisin B1	5 $\pm$ 2	3 $\pm$ 1
D-Erythrospingosine	15 $\pm$ 6	53 $\pm$ 7
D-Erythrospingosine + fumonisin B1	12 $\pm$ 3	48 $\pm$ 3
L-Erythrospingosine	15 $\pm$ 6	55 $\pm$ 8
L-Erythrospingosine + fumonisin B1	13 $\pm$ 4	51 $\pm$ 9
Sphinganine	13 $\pm$ 2	49 $\pm$ 2
Sphinganine + fumonisin B1	11 $\pm$ 1	50 $\pm$ 11
Dimethylsphingosine	30 $\pm$ 5	83 $\pm$ 10
Dimethylsphingosine + fumonisin B1	28 $\pm$ 6	80 $\pm$ 12
Stearylamine	10 $\pm$ 2	25 $\pm$ 5
Stearylamine + fumonisin B1	12 $\pm$ 4	21 $\pm$ 3

absolutely rule out the possibility that PKC may be involved in this apoptotic process, for several reasons. First, both D- and L-erythrospingosine can effectively induce apoptosis in Hep3B cells and, since these two sphingosine stereoisomers have been shown to inhibit PKC activity with similar potency *in vitro* and in cells [20], this implies that modulation of PKC activity may be a critical step in sphingosine-induced apoptosis. Secondly, it is possible that the concentration of sphingosine used in our study (20 μ M) is too high for PMA (at 10 nM) to overcome, although PMA at 100 nM is known to be able to protect cells from tumour necrosis factor α (TNF α)- and C₂-ceramide-induced apoptosis [22]. However, high concentrations of PMA (> 100 nM) alone induced apoptosis in Hep3B cells (results not shown). Thus direct analysis of the PKC activity during apoptosis induced by sphingolipids is required to clarify the functional role of PKC in this process.

Since sphingolipids may be further metabolized into ceramide, a novel lipid mediator of apoptosis, in cultured cells, and since many apoptosis-inducing signals (including TNF α , chemotherapeutic drugs, Fas protein and ionizing radiation) have been demonstrated to activate apoptosis via ceramide formation [23,24], we investigated whether these sphingolipids might be converted into ceramide in order to elicit their cytotoxic effects. We used the fungal agent fumonisin B1 (20 μ M) to block the activity of ceramide synthase and therefore inhibit the conversion of sphingosine into ceramide, and we found that fumonisin B1 could not attenuate the apoptosis induced by sphingolipids (Table 3). These data suggest that sphingolipids act independently of ceramide in the induction of apoptosis in Hep3B cells. Furthermore, an analogue of sphingosine, dimethylsphingosine, which cannot be metabolized into ceramide in cells, also has a potent cytotoxic effect on Hep3B cells. These results indicate that ceramide is not involved in sphingolipid-induced apoptosis.

Sphingosine-induced apoptosis is blocked by the general caspase inhibitor ZVAD-FMK

We next examined whether caspase-family proteases are involved in the apoptotic process induced by sphingosine. Because all of the ICE-like proteases can cleave substrates that have an aspartic

Table 4 Inhibition of sphingosine-induced apoptosis in Hep3B cells by the caspase inhibitor ZVAD-FMK

Hep3B cells were preincubated with various concentrations of the caspase inhibitor ZVAD-FMK for 2 h and were then treated with 20 μ M sphingosine for another 24 h. Apoptotic cells were determined and expressed as a percentage of the total cell number measured, as described in Table 1. Results from three independent experiments are shown as means $\pm$ S.E.M.

Treatment	Apoptotic cells (% of total cell number)
Control	4 $\pm$ 2
Sphingosine	51 $\pm$ 10
ZVAD-FMK (10 μ M)	3 $\pm$ 1
ZVAD-FMK (50 μ M)	4 $\pm$ 1
Sphingosine + ZVAD-FMK (10 μ M)	13 $\pm$ 6
Sphingosine + ZVAD-FMK (50 μ M)	5 $\pm$ 4

Figure 2 Inhibition of sphingosine-induced DNA fragmentation by ZVAD-FMK

Cells cultured in DMEM/F12 medium containing 0.5% (v/v) FCS were preincubated with 50 μ M ZVAD-FMK for 2 h and were then treated with 20 μ M sphingosine for another 24 h. DNA samples were extracted from cells as described in the Materials and methods section, subjected to agarose-gel electrophoresis, stained with ethidium bromide and visualized under UV light. Lane M contains ϕ X174 DNA *Hae*III digest molecular mass markers.

acid in the P1 position, we investigated whether the general caspase inhibitor ZVAD-FMK, which has been demonstrated to block the activity of a number of caspases, could inhibit sphingosine-triggered apoptosis. As shown in Table 4, ZVAD-FMK attenuated sphingosine-triggered apoptosis in a concentration-dependent manner. In addition, we examined the effect of ZVAD-FMK on sphingosine-activated DNA fragmentation, another biological hallmark of apoptosis. Figure 2 demonstrates that sphingosine at 20 μ M induced DNA fragmentation in Hep3B cells, and that pretreatment with 50 μ M ZVAD-FMK significantly attenuated sphingosine-triggered DNA fragmentation in these cells. Thus our results indicate that caspase activation may play an important role in the induction of apoptosis by sphingosine.

Sphingosine induces the catalytic activity of caspase-3-like proteases in Hep3B cells

To clarify further the specific caspases activated during apoptosis triggered by sphingosine, we investigated the effects of another two caspase inhibitors on sphingosine-induced apoptosis. YVAD-CMK is a specific ICE (caspase-1) inhibitor, while DEVD-FMK is known to be a specific inhibitor of the CPP32-

Table 5 Specific attenuation of sphingosine-induced apoptosis by the caspase-3 inhibitor DEVD-FMK

Cells were preincubated with the caspase-3 inhibitor DEVD-FMK or the caspase-1 inhibitor YVAD-CMK (50 μ M) for 2 h, and then treated with 20 μ M sphingosine for another 24 h. Apoptotic cells were determined as described in Table 1.

Treatment	Apoptotic cells (% of total cell number)
Control	2 $\pm$ 1
Sphingosine	48 $\pm$ 9
DEVD-FMK	3 $\pm$ 2
YVAD-CMK	5 $\pm$ 1
Sphingosine + DEVD-FMK	10 $\pm$ 5
Sphingosine + YVAD-CMK	45 $\pm$ 8

Figure 3 Effects of sphingosine and etoposide on PARP proteolysis

Cells were treated with 20 μ M sphingosine (Sph) or 50 μ M etoposide (Eto) in DMEM/F12 medium containing 0.5% (v/v) FCS for different time intervals, and then harvested in a lysis buffer. Cleavage of PARP was investigated by Western blot analysis as described in the Materials and methods section. Positions of molecular mass markers (kDa) are indicated on the right.

like (caspase-3-like) proteases [25]. Table 5 shows that apoptosis induced by sphingosine was inhibited by DEVD-FMK, but not by YVAD-CMK, at a concentration of 50 μ M. These results suggest that sphingosine activates caspase-3-like proteases in Hep3B cells. However, it should be noted that the concentration of DEVD-FMK used in our study (50 μ M) only partially blocked apoptosis triggered by sphingosine. Although higher concentrations of DEVD-FMK (400 μ M) completely inhibited sphingosine-induced apoptosis (results not shown), other caspases may be non-specifically inhibited by DEVD-FMK at this concentration. Therefore it is possible that caspases other than caspase-3 are also activated in this apoptotic process.

To confirm the results obtained with the synthetic inhibitors, the increase in caspase-3 activity was analysed by two additional experimental approaches. First, we investigated the proteolytic cleavage of PARP in sphingosine-treated cells. PARP was the first identified *in vivo* substrate for caspases, and caspase-3 was reported to cleave PARP with the highest efficiency *in vitro* [26]. As shown in Figure 3, native PARP (molecular mass 116 kDa) was cleaved to give a typical fragment of 85 kDa in sphingosine-treated cells, but not in control cells. In addition, Hep3B cells were cultured with etoposide (50 μ g/ml), a chemotherapeutic agent that induces apoptosis and caspase-3 activation in a variety of cancer cell lines [27], as a positive control for the study of the proteolytic activity of caspase-3. Indeed, cleavage of PARP was clearly detected in etoposide-treated Hep3B cells. However, kinetic studies indicated that etoposide activated PARP proteolysis more quickly than did sphingosine, in that the 85 kDa

Figure 4 Inhibition of PARP proteolysis in sphingosine-treated Hep3B cells by DEVD-FMK

Cells were incubated with 20 μ M sphingosine in the presence or absence of 50 μ M DEVD-FMK for 6 h, and were then harvested in a lysis buffer. Determination of protein concentrations and investigation of the expression and proteolysis of PARP was performed as described in the legend to Figure 3. Positions of molecular mass markers are indicated on the left.

Table 6 Induction of caspase-3 catalytic activity by sphingosine

Cells were preincubated with 50 μ M DEVD-FMK for 2 h and then treated with sphingosine (20 μ M) or etoposide (50 μ g/ml) for another 12 h. Cells were harvested and the catalytic activity of caspase-3 was determined by using an enzymic assay kit as described in the Materials and methods section. Results are expressed as means $\pm$ S.E.M. for three separate experiments.

Treatment	Absorbance (% of control)
Control	100
DEVD-FMK	98 $\pm$ 5
Sphingosine	250 $\pm$ 20
Sphingosine + DEVD-FMK	105 $\pm$ 15
Etoposide	407 $\pm$ 22
Etoposide + DEVD-FMK	101 $\pm$ 12

fragment appeared 3 h after etoposide addition, while the cleavage of PARP was not observed at this time point in sphingosine-treated cells. Figure 4 demonstrates that pretreatment with DEVD-FMK inhibited sphingosine-activated PARP proteolysis.

In a second approach, we used the substrate DEVD-pNA to analyse the catalytic activity of caspase-3 directly. Activation of caspase-3 leads to the cleavage of DEVD-pNA, release of the chromophore pNA and an increase in absorbance at 405 nm. Table 6 shows that a 2.5-fold increase in absorbance was detected in Hep3B cells after treatment with 20 μ M sphingosine for 12 h, and that DEVD-FMK significantly attenuated the proteolytic activity of caspase-3. Under the same experimental conditions, incubation with 50 μ g/ml etoposide led to a 4-fold increase in absorbance, and this increase in caspase-3 activity was also blocked by the addition of DEVD-FMK.

DISCUSSION

Caspase-3, a member of the ICE family, was cloned from a T-lymphocyte cell line, and is expressed in all tissues examined so far [28]. Recent studies have suggested that caspase-3 may play a central role in regulating and executing mammalian cell apoptosis, because the activation of this protease was shown to be an early and essential step in multiple apoptotic signalling pathways triggered by different apoptotic signals [29,30]. Our data indicate that caspase-3-like proteases are key regulators in the apoptotic process induced by sphingosine. Although several sphingolipids (such as sphingosine, sphingosine 1-phosphate and *N,N*-dimethylsphingosine) have been demonstrated to elicit apoptotic effects in a number of cancer cell lines [11,31], the involvement of caspase-3-like proteases in sphingosine-activated

apoptosis is a new finding. In the study of the induction of the catalytic activity of caspase-3 by sphingosine, cells treated with etoposide were always included to verify the experimental results. Previous work demonstrated that this drug is a very potent caspase-3 activator and inducer of apoptosis in many cancer cell lines, and etoposide-induced caspase-3 activation was effectively inhibited by Asp-Glu-Val-Asp aldehyde [32]. However, it should be emphasized that activation of caspase-3 is an early event in the etoposide-mediated apoptotic pathway, whereas such activation is a relatively later step in the apoptotic pathway induced by sphingosine.

Cain et al. [33] demonstrated that YVAD-CMK inhibited apoptosis of rat hepatocytes induced by transforming growth factor β 1 and staurosporine, and these results suggested that an ICE-like protease is involved in the induction of apoptosis in these cells. Given the results with human hepatoma cells in the present study, it is suggested that different apoptotic signals may show different preferences in the activation of caspases. Furthermore, a caspase may act as an inducer caspase in one apoptotic pathway, while it may act as an executor caspase in another. Our data also suggest that sphingosine may activate a number of caspases through a caspase/protease cascade in Hep3B cells. This result may not be unexpected, since activation of different caspases in cells, either simultaneously or sequentially, by different apoptotic stimuli has been clearly demonstrated [34,35]. Further work is required to identify the caspases, other than caspase-3, that participate in sphingosine-induced apoptosis. However, since human hepatocytes have been reported to express large amounts of caspase-3 protein [36], it seems reasonable to speculate that this is one of the major proteases activated in sphingosine-treated hepatoma cells.

Evidence has accumulated that sphingolipids and their metabolites are involved in the regulation of cell proliferation, differentiation and apoptosis [37]. However, it should be noted that the signalling pathways activated by different sphingolipids may be different, and that many sphingolipid-related phenomena are expressed in a cell-type-dependent manner. For example, ceramide generated by the hydrolysis of membrane-associated sphingomyelin induces apoptosis in Jurkat lymphocytes, but sphingosine 1-phosphate, a metabolite of ceramide, prevents ceramide-triggered apoptosis in these cells [38]. Similarly, different outcomes may be found when the same sphingolipid is added into the culture medium of different cell lines. For instance, sphingosine 1-phosphate stimulates cell proliferation of fibroblasts, whereas it induces growth arrest in breast cancer cells [39,40]. In addition, this sphingolipid can trigger apoptosis in HL60 and Hep3B cells [11,12]. Despite questions regarding their mechanisms of action, recent data indeed indicate that sphingolipids are potent anti-tumour agents. Several important features of the anti-tumour activity of sphingolipids should be noted. First, sphingolipids and their bioactive metabolites elicit potent anti-tumour effects *in vivo*, similar to those seen *in vitro* [41]. Secondly, sphingolipids can effectively and specifically inhibit the chemotactic motility and invasiveness of tumour cells [42]. Thirdly, exogenously added sphingolipids exert low toxicity on normal fibroblasts or normal human umbilical-vein endothelial cells at concentrations that trigger apoptosis in different cancer cell lines [12]. Finally, and most importantly, sphingolipids can effectively inhibit the growth of cancer cells that are resistant to anti-cancer drugs, and which express high levels of P-glycoprotein, both *in vitro* and *in vivo* [43]. This implies that sphingolipids may be useful in the therapy of human cancers that are unresponsive to classic chemotherapeutic drugs.

The signalling roles of different sphingolipids are currently receiving much attention in biomedical research fields. A critical

issue to be addressed is whether sphingosine is an apoptotic inducer under physiological conditions. Although our results show that the apoptosis-inducing action is not specific to sphingosine, and may extend to L-erythrosphingosine or stearylamine, a functional role for this sphingolipid in mediating apoptotic signals from different external insults cannot be excluded. Previous studies have demonstrated that sphingosine is an endogenous modulator that mediates apoptotic signals induced by TNF α in neutrophils and cardiac myocytes [44,45]. It has also been reported that sphingosine can markedly potentiate the induction of apoptosis by ceramide when present at sub-micromolar levels, which may also have physiological significance [46]. Additionally, it should be noted that sphingosine was added exogenously in our study, and thus the amount of sphingosine taken up by the Hep3B cells was not known. It is well known that sphingosine has a low pK_a (about 6.7), and is present as both neutral and ionized forms at physiological pH [20]. As a charged species, it would be predicted to have difficulty in crossing the plasma membrane. Therefore, whether the apoptotic effect of sphingosine observed in the present study represents a physiological or pharmacological action of this sphingolipid requires further characterization. In addition to their anti-tumour effects, sphingolipids also participate in the regulation of various physiological processes, including platelet aggregation, neutrophil-mediated inflammatory responses and retraction of neurites in neuronal cells [47–49]. Therefore the characterization of specific signalling pathways triggered by specific sphingolipids is important for the understanding of the different biological function of sphingolipids.

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