Bacterial ceramides and sphingophospholipids induce apoptosis of human leukaemic cells

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The genus *Sphingobacterium*, whose members are Gram-negative non-fermentative rods, possesses ceramides and related sphingophospholipids (SPLs) with isoheptadecasphinganine and 2-hydroxy or non-hydroxy isopentadecanoic acid. This paper reports evidence that ceramides isolated from *Sphingobacterium spiritivorum* ATCC 33861 induce endonucleolytic DNA cleavage in human myeloid leukaemia HL-60 cells *in vitro*, which is the primary characteristic biochemical marker for apoptosis or programmed cell death. Ceramides and SPLs also induced DNA fragmentation and caspase-3 activation, followed by changes in morphology, such as alterations in the size of nuclei and cells, and cell cycle shortening. Apoptotic activity correlated with the ceramide structure. Ceramide with a 2-hydroxy fatty acid showed stronger apoptotic activity than ceramide with a non-hydroxy fatty acid. Furthermore, the major five SPLs (ceramide phosphorylethanolamine-1 and -2, ceramide phosphorylinositol-1 and -2, and ceramide phosphorylmannose-1) showed apoptosis-inducing activity in HL-60 cells, indicating that the ceramide moiety of the SPLs plays a crucial role as the intracellular second messenger but that their hydrophilicity is less important in this regard. The hydrophilic part of SPLs may play a role in other cellular response systems. The involvement of Fas antigen was implicated in the apoptotic event since Fas antigen expression was observed after 3 or 4 h stimulation of HL-60 cells and cell cycle shortening with bacterial ceramides. However, a time-course study for caspase-3 activation indicated maximal activity at 1 h after stimulation with bacterial ceramides, suggesting that two (or possibly more) mechanisms of signal transduction, Fas-dependent and Fas-independent, may be involved. Fas antigen expression and caspase-3 activation by five kinds of SPLs were observed after 3 or 4 h. These results indicate that there is a difference in the response of HL-60 cells to bacterial ceramides and SPLs.

INTRODUCTION

Ceramide is the hydrophobic part of sphingophospholipids (SPLs) and sphingoglycolipids, both of which are ubiquitous components of eukaryotic cell membranes (Parton, 1994; Rothberg et al., 1992; Schnitzer et al., 1996). Sphingolipids play diverse physiological roles in mammalian cells, such as in cell differentiation (Furuya et al., 1995; Goodman & Mattson, 1996; Harel & Futerman, 1993; Okazaki et al., 1990), proliferation (Hannun et al., 1986; Ito & Horigome, 1995; Schwarz & Futerman, 1997) or receptor-mediated endocytosis (Okazaki et al., 1989). Ceramide itself also has been shown to be a second messenger (Lavie et al., 1997; Okazaki et al., 1989) and to play a crucial role in the induction of apoptosis, via activation of cellular signal transduction systems (Linaric & Hannun, 1994; Liu & Anderson, 1995; Okazaki et al., 1994).

Until recently, the occurrence of ceramide or sphingolipid in bacteria has been considered to be extremely rare. In 1982, we first reported the occurrence of ceramides and SPLs possessing novel branched, long-chain bases and branched-chain fatty acid as a major lipid component in *Pseudomonas*-like Gram-negative, non-fermentative rods (CDC type 2-b, later termed the *Sphingobacterium* genus) (Yano et al.,...
1982). The major molecular species were identified as N-isopentadecanoyl isoheptadecasphinganine and its 2-hydroxy fatty acid analogues (Yano et al., 1983). Eukaryotic ceramides have fatty acyl chains with 16–26 carbon atoms and have been reported to induce apoptosis of undifferentiated cells, after membrane sphingomyelin hydrolysis by acidic sphingomyelinase (Wiegmann et al., 1994; Yang et al., 1993). However, since mammalian ceramides are poorly permeable in the cell membrane, synthetic and shorter-chain ceramide analogues such as acetyl (C2) or hexanoyl (C6) ceramides have been used to demonstrate their physiological function when they are administered exogenously (Allan, 2000; Hartfield et al., 1997).

Since the bacterial ceramides possess unique structures with branched, long-chain base and fatty acid (Nakayama, 2000), we have investigated their ability to induce apoptosis in HL-60 cells in vitro, when administered exogenously. N-2-Hydroxyisopentadecanoyl isoheptadecasphinganine was more than twice as active as mammalian ceramide in inducing typical features of apoptotic cell death. Furthermore, SPLs such as a novel ceramide phosphorylmannose (cerPM-1), ceramide phosphorylethanolamines (cerPE-1 and cerPE-2) and ceramide phosphorylinositol cerPI-1 and cerPI-2), all of which are good chemotaxonomic markers unique to the genus *Sphingobacterium*, were found to have similar apoptotic activity. This is the first report describing that bacterial ceramides and sphingolipids induce programmed cell death in mammalian cells when administered exogenously.

**METHODS**

**Isolation of bacterial ceramides and SPLs.** Strains of *Sphingobacterium* species were supplied by Dr Eiko Yabuuchi, Aichi Medical University, Aichi, Japan. *Sphingobacterium spiritivorum ATCC 33861* was grown in a medium containing 1% (v/v) polypeptone (Nissui) and 0.5% (v/v) yeast extract (Difco), pH 7.0, at 30 °C for 30 h with vigorous shaking (OD610 > 0.6). After harvest of the autoclaved cells by centrifugation, lipids were extracted by a modification of the method of Folch et al. (1959) using chloroform/methanol (2 : 1, v/v), successively. To decompose the alkali-labile acylceramide, lipids were hydrolysed with 0.5 M KOH in methanol/chloroform (1 : 1, v/v) at 37 °C for 3 h with shaking. After neutralization, the resultant alkali-stable lipids were extracted with chloroform/methanol (2 : 1, v/v) and then separated by preparative TLC on silica-gel G (Analtec) with the acidic solvent system chloroform/methanol/acetic acid/water (100 : 20 : 12 : 5, by vol.). Free ceramides and alkali-stable SPLs were separated as reported previously (Yano et al., 1982, 1983). To reveal the ceramide band, thin-layer plates were exposed to iodine vapour after development. The ceramide bands were marked and then the gels were scraped to elute the lipid through a small glass column.

The TLC purification was repeated until a single spot was obtained. Analysis and identification of bacterial ceramides and SPLs were performed essentially according to the previous report (Yano et al., 1983), by gas chromatography/mass spectrometry (GC/MS) of each constituent and fast atom bombardment-mass spectrometry (FAB/MS) of the intact molecule.

**Cell culture.** Human myeloid leukaemia HL-60 cells (from the ATCC) were maintained in RPMI 1640 medium (Nissui) supplemented with 20% (v/v) fetal bovine serum (FBS) and 2 mM L-glutamine. Human promonocytic U-937 cells (ATCC) and human leukaemia Jurkat cells (Dainippon Pharmaceutical) were maintained in RPMI 1640 medium supplemented with 10% (v/v) FBS and 2 mM L-glutamine. For apoptotic experiments, cells were resuspended in RPMI 1640 medium supplemented with 1% (v/v) FBS and 2 mM L-glutamine.

**Co-culture of HL-60 cells and bacteria or heat-inactivated bacteria.** *S. spiritivorum* was grown and prepared as above to yield a suspension of OD600 > 0.6. Bacterial cells were collected by centrifugation and passed through a 0.45 μm disposable filter. This bacterial suspension (100 μl) was added to HL-60 cells (5 x 10⁶ cells) and these were incubated for 1 h at 37 °C. HL-60 cells were washed once with phosphate-buffered saline (PBS) and further cultured in RPMI 1640 medium containing 1% (v/v) FBS for 24 h at 37 °C in 5% CO₂. We then analysed the cell cycle by flow cytometry. Heat inactivation of *S. spiritivorum* was performed for 30 min at 65 °C. After cooling to room temperature, the heat-inactivated bacteria (100 μl) were added to HL-60 cells (5 x 10⁵ cells) and cultured in RPMI 1640 medium containing 1% (v/v) FBS for 24 h at 37 °C in 5% CO₂. We then analysed the cell cycle by flow cytometry.

**Measurement of the cell cycle by flow cytometry.** Cell cycle analysis was done according to a standard protocol (Taylor, 1980). Briefly, aliquots of 2.5 x 10⁶ cells were washed twice with PBS and fixed in 70% ethanol for 5 min on ice. The cell pellets were washed twice with PBS, and resuspended in PBS containing RNase A (1 μg ml⁻¹, Nippon Gene), and incubated for 30 min at 37 °C. The cells were stained with propidium iodide at 20 μg ml⁻¹ for 5 min and analysed by flow cytometry. The percentage of apoptotic cells was calculated using the internal software system of the FACSscan (Becton Dickinson).

**Flow cytometric analysis of Fas antigen expression.** Cell-surface expression of Fas antigen was determined by flow cytometric analysis (Yonehara et al., 1989). HL-60 cells (5 x 10⁶ cells ml⁻¹) were seeded in 35 mm plastic dishes and treated with sphingolipids [500 nM dissolved in ethanol/dodecane (98:2, v/v)], in RPMI 1640 medium containing 1% (v/v) FBS. At the indicated time points, the cells were harvested, washed once with PBS, and reacted on ice for 1 h with 0.1 ml PBS containing 1% (v/v) FBS, 0.02% (v/v) NaN₃ and anti-Fas IgM antibody (20 μg ml⁻¹; MBL, Nagoya, Japan). Cells were washed twice with PBS, then for an additional 1 h with 0.1 ml PBS containing 0.02% (v/v) NaN₃ and 10 μg ml⁻¹ FITC-conjugated goat anti-mouse IgM (Cappel). After washing once with PBS, cellsurface-bound FITC-anti-mouse IgM was analysed by flow cytometry. The percentage of cells expressing Fas antigen was calculated from fluorescence intensity using the internal software system of the FACSscan.

**Apoptotic morphology.** HL-60 cells (5 x 10⁶ cells ml⁻¹) were seeded in 35 mm plastic dishes and treated with sphingolipids [0–500 nM dissolved in ethanol/dodecane (98:2, v/v)], in RPMI 1640 medium containing 1% (v/v) FBS. At the indicated time points, the cells were harvested and fixed with 1% (v/v) glutaraldehyde in PBS for 1 h. After washing once with PBS, the cells were stained with 0.2 mM Hoechst 33342 for 10 min in the dark. Chromatin condensation was examined by fluorescence microscopy. Experiments were performed at least in triplicate.

**Analysis of DNA fragmentation.** DNA fragmentation was analysed by agarose gel electrophoresis (Gorczyna et al., 1993). After the appropriate period of incubation (0–4 h) of HL-60 cells (5 x 10⁶ cells ml⁻¹) with 500 nM sphingolipids, cells were harvested by centrifugation at 400 g for 5 min, washed with PBS, and incubated with digestion buffer [0.1 M NaCl, 10 mM Tris/HCl (pH 8.0), containing 25 mM EDTA and 0.5% (w/v) SDS]. Proteinase K
(0·2 mg ml⁻¹; Takara) was then added and the incubation was continued at 50 °C overnight. The cellular lysates were centrifuged at 13 000 g for 20 min to separate the low-molecular-mass DNA from the chromatin. Fragmented DNA was extracted from the supernatant by phenol/chloroform/isoamyl alcohol (25:24:1, by vol.). The upper aqueous layer was placed in a fresh tube and 0·5 vol. 7·5 M ammonium acetate solution followed by 2 vols absolute alcohol (4 °C) were added to precipitate the DNA. After washing with 70 % (v/v) ethanol, to remove residual RNA, 0·1 % (w/v) SDS solution containing 0·625 mg ml⁻¹ RNase A was added, and the sample was incubated for 4 h at 37 °C. After repeated extraction with phenol/chloroform/isoamyl alcohol and ethanol precipitation, the DNA was dissolved in TE buffer (10 mM Tris/HCl pH 8·0, 1 mM EDTA), loaded on 1·5 % (w/v) agarose gel with ethidium bromide and electrophoresed for 30 min at 100 V. Bands were visualized by UV illumination.

**Estimation of activities of ICE (-like) and CPP32/Yama (-like) proteases (caspases) (Shimizu et al., 1996).** HL-60 cells (5 × 10⁵ cells ml⁻¹) were treated with ceramides or sphingophospholipids [500 nM dissolved in ethanol/dodecane (98:2, v/v)], in RPMI 1640 medium containing 1 % (v/v) FBS. After 0·5, 1, 2, 3 and 4 h, cells were washed three times with PBS, suspended in 50 mM Tris/HCl (pH 7·4), 1 mM EDTA, 1 mM EGTA and 0·2 % (v/v) Triton X-100 and incubated at 37 °C for 10 min. Cell lysates (supernatants) were prepared by centrifugation at 25 000 g for 3 min, and cleared lysates containing 40 µg protein were incubated with 50 µM enzyme substrate Ac-Try-Val-Ala-Asp-MCA (Peptide Institute, Osaka, Japan) and Ac-Asp-Glu-Val-Asp-MCA (Peptide Institute) at 37 °C for 1 h. Levels of released 7-amino-4-methylcoumarin (AMC) were measured with a spectrofluorometer (Hitachi F-4000) with excitation at 380 nm and emission at 460 nm. Excitation and emission slit widths were adjusted to 10 mm and 20 mm, respectively. One unit was defined as the amount of enzyme required to release 0·22 nmol AMC min⁻¹ at 37 °C.

**Inhibition of apoptosis by caspase inhibitors.** HL-60 cells (5 × 10⁵ cells ml⁻¹) were treated with 250 µM caspase-1 specific inhibitor.

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**Fig. 1.** Structures of ceramides and SPLs from *S. spiritivorum*. The ceramide of cerPE-1, cerPM-1 and cerPI-1 is the same as that of cer-A, and the ceramide of cerPE-2 and cerPI-2 is the same as that of cer-B.
inhibitor (Ac-Tyr-Val-Ala-Asp-H, Peptide Institute) or 250 μM caspase-3 specific inhibitor (Ac-Asp-Glu-Val-Asp-H, Peptide Institute) for 2 h at 37°C in 5% CO2. HL-60 cells were then treated for 4 h with ceramide or SPLs [500 nM dissolved in ethanol/dodecane (98:2, v/v)], in RPMI 1640 medium containing 1% (v/v) FBS. The cells were harvested and washed once with PBS. After this, they were fixed with 70% (v/v) ethanol for 5 min on ice, stained with propidium iodide and assayed by flow cytometry, as described above.

**Statistical analysis.** Data are expressed as mean values ± SD from 3–9 experiments. Significant difference was evaluated by an unpaired Student’s t-test.

## RESULTS

**Structures of ceramides and SPLs from *Sphingobacterium* species**

The structures of bacterial ceramides from *Sphingobacterium* species have been reported already (Yano et al., 1982, 1983). Recently, we studied the composition of membrane lipid from the genus *Sphingobacterium* and showed that it is unique in possessing various SPLs and ceramides, besides glycerophospholipids (data not shown). Five major cellular SPLs were isolated and identified from *S. spiritivorum*, the type species of the genus *Sphingobacterium*. These were identified as cerPE-1, cerPE-2, cerPM-1, cerPI-1 and cerPI-2 (Fig. 1). The ceramide of cerPE-1, cerPM-1 and cerPI-1 was composed of isoheptadecasphinganine (iso-C17:0) and isopentadecanoic acid (iso-C15:0), whereas that of cerPE-2 and cerPI-2 was composed of isoheptadecasphinganine (iso-C17:0) and 2-hydroxyisopentadecanoic acid (2-OH iso-C15:0). CerPM-1 is a novel SPL within eukaryotic and prokaryotic cells and may be a good candidate for a specific chemotaxonomic marker of the genus *Sphingobacterium*. The structures of each bacterial ceramide and SPL from *S. spiritivorum* (determined by GC/MS and FAB/MS) are shown in Fig. 1. TLC chromatograms are shown in Fig. 2. A negative FAB/MS spectrum of ceramide-A (cer-A) showed a quasi-molecular ion (M–H) at m/z 510, indicating isopentadecanoyl isoheptadecasphinganine (M, 511), whilst that of ceramide-B (cer-B) showed a (M–H)– ion at m/z 526, 16 mass units higher than the former due to the introduction of one oxygen atom, indicating 2-hydroxyisopentadecanoyl isoheptadecasphinganine (M, 527), exclusively.

**Apoptosis induction by bacterial ceramide in different lymphoma cell lines**

To examine whether bacterial ceramide induced lymphoma apoptosis, we analysed the cell cycle by flow cytometry. U-937 cells did not exhibit apoptosis at the ceramide concentrations tested (Fig. 3). In contrast, when Jurkat cells were incubated for 4 h with 1 or 10 μM cer-A, the proportion of apoptotic cells increased in a dose-dependent manner (Fig. 3). Moreover, when HL-60 cells were incubated for 4 h with 500 nM cer-A, the proportion of apoptotic cells increased. The ratio of apoptotic cells

![Fig. 2. TLC of ceramides and SPLs from *S. spiritivorum*. Chloroform/methanol/acetic acid/water (100:20:12.5, by vol.) was used as solvent system. a, crude lipid; b, cer-A; c, cer-B; d, cerPE-1; e, cerPE-2; f, cerPM-1; g, cerPI-1; h, cerPI-2.](image)

![Fig. 3. Percentage of apoptotic cells induced by cer-A in Jurkat, U-937 and HL-60 cells. Cells (2.5 × 10⁶ cells in a 35 mm dish), prepared as described in Methods, were incubated for 4 h with different concentrations of cer-A. For Jurkat cells, the proportion of apoptotic cells was: control, 8.08 ± 0.23%; treated with 1 μM cer-A, 42.43 ± 1.16%; treated with 10 μM cer-A, 60.95 ± 0.46%. When HL-60 cells were treated with 500 nM cer-A, 64.41 ± 6.1% of the cells were apoptotic. Data represent the mean ± SD of three experiments. *, P < 0.001 versus control.](image)
induced with cer-A was highest in HL-60 cells. We therefore used HL-60 cells for subsequent experiments.

It was unclear whether S. spiritivorum cells are capable of inducing apoptosis directly since ceramide resides in the inner membrane in these Gram-negative bacteria. In order to explore this, HL-60 cells were incubated with S. spiritivorum or heat-inactivated S. spiritivorum. Neither bacterial sample induced apoptosis. However, 10 μg ml⁻¹ crude lipid from S. spiritivorum induced a marked apoptosis (21.45 ± 0.17 % apoptotic cells) in HL-60 cells after incubation for 24 h.

**Morphological changes in HL-60 cells with exogenous sphingolipids**

To observe specific apoptotic changes of nuclei in HL-60 cells, the cells were treated with 500 nM cer-A for 4 h, stained with the DNA-labelling fluorochrome Hoechst 33258, and examined by phase-contrast and fluorescence microscopy. As shown in Fig. 4, many cells showed fragmented and/or condensed nuclei. Membrane blebbing and condensation and a marked shrinkage of the cells were also observed, showing that a typical apoptosis event occurred.

**Apoptosis-inducing activity of bacterial ceramides and SPLs**

To explore the kinetics of the apoptosis process, time-course studies of DNA fragmentation induced by ceramides or SPLs were undertaken (Fig. 5). The early stage of nucleosomal fragmentation was clearly observed in HL-60 cells after 1 or 2 h incubation with 500 nM of ceramides or SPLs, followed by progressive DNA fragmentation during the 4 h incubation. The relative activities for inducing apoptosis of HL-60 cells were compared for cer-A, cer-B and mammalian ceramide (500 nM in each case), as shown in Fig. 6. Based on the relative ratio of apoptotic cells analysed by flow cytometry, cer-B showed the highest activity after incubation for 1–4 h, followed by cer-A. In contrast, under this condition, the mammalian ceramide showed only a small apoptotic activity with HL-60 cells: its activity was less than half that of cer-B after 4 h incubation.

Little or no information has been reported on the apoptosis-inducing activity of exogenous SPLs, although C₂ or C₆ synthetic ceramide has been shown to be capable of inducing apoptosis when added exogenously (Sawai et al., 1995). The relative activities for inducing apoptotic alterations in HL-60 cells treated with 500 nM of each of the five major Sphingobacterium SPLs were examined by flow cytometry (Fig. 7). The effects of cer-A and cer-B were also examined for comparison. Both ceramides and the five SPLs induced clear apoptosis of HL-60 cells. No significant difference was observed in the potency of apoptosis induction between cerPE-1 and cerPE-2, or between cerPI-1 and cerPI-2. CerPE-1 and cerPI-1 include cer-A in their structure, whilst cerPE-2 and cerPI-2 include cer-B (see Fig. 1). The observed apoptotic activity of the SPLs did not correlate with the presence or absence of the 2-hydroxy fatty acid. Also, apoptotic activity did not appear to relate to the presence of diacyl phospholipids, such as phosphatidylethanolamine or phosphatidylcholine.

**Apoptosis induction in HL-60 cells by iso-C15:0 fatty acid and iso-C17:0 long chain sphinganine base**

Cer-A, cer-B and the five major SPLs, which all elicit apoptosis, contain iso-C15:0 fatty acid and iso-C17:0 long-chain sphinganine base as a common component. To determine the minimum common structure necessary for potent apoptotic induction, the activities of iso-C15:0 fatty acid and iso-C17:0 long-chain base were examined by DNA fragmentation analysis, using 1.5 % agarose gel electrophoresis. As shown in Fig. 8, a slight DNA fragmentation was observed when HL-60 cells were treated with 500 nM iso-C17:0 sphinganine base for 2 h, but not with iso-C15:0 fatty acid. These results suggest that iso-C17:0 sphinganine base is the primary component of ceramide and/or SPL structure required to induce apoptosis.

**Expression of Fas antigen**

The Fas receptor system has been extensively studied as a model of apoptosis, and cross-linking of the Fas receptor with Fas ligand or specific agonist antibodies results in
rapid programmed cell death. Expression of Fas antigen on HL-60 cells treated with cer-A, cer-B or SPLs for 4 h was assayed. As shown in Fig. 9, the expression of Fas antigen was observed at 2 h after HL-60 cells were treated with 500 nM cer-A, cer-B or SPLs, and then continued to increase with time up to 4 h.

Estimation of caspase activity

Since ceramide-induced apoptosis has been reported to involve a caspase-dependent step, the activities of caspase-1 and caspase-3 in HL-60 cells treated with cer-A, cer-B or
SPLs were estimated. As shown in Fig. 10, a marked increase in caspase-3 activity was demonstrated soon after HL-60 cells were treated with cer-A or cer-B. Activity reached a maximum at 1 h before declining. The kinetics of caspase-3 expression did not correlate well with that of the ceramide-induced DNA fragmentation. These results suggest that caspase-3 might be activated in the early stages, but not the later stages, of apoptosis of HL-60 cells treated with ceramides. In contrast to the pattern observed with free ceramides, the five SPLs resulted in an increase in caspase activity at around 3 or 4 h. However, the activity was not strong in comparison with that seen with cer-A or cer-B. No elevation of caspase-1 activity was observed in the cells treated with cer-A, cer-B or any of the five SPLs during the experimental period (Fig. 10).

To confirm the involvement of caspases in ceramide-induced apoptosis, the effects of caspase-3 inhibitor (Ac-DEVD-CHO) and caspase-1 inhibitor (Ac-YVAD-CHO) were examined. Each inhibitor was added 2 h before treatment with ceramides and SPLs. As shown in Fig. 11, the inhibitor of caspase-3 showed a significant suppression of apoptosis for HL-60 cells treated with cer-A, cer-B or the SPLs, whereas the inhibitor of caspase-1 showed no effect. We concluded that caspase-3 activation was linked with the induction of apoptosis of HL-60 cells by cer-A, cer-B or the SPLs.

**DISCUSSION**

Apoptosis is a form of cell death induced in eukaryotic cells by exogenous stresses (Kondo *et al.*, 2000a, b; Verheij *et al.*, 1996), chemical substances (Decaudin *et al.*, 1997), toxins (Iwata *et al.*, 2002) and endocrine stimulation (Saito & Kurasaki, 2000). However, differing from necrosis, apoptosis is a programmed cell death contributing to physiological functions such as embryogenesis (Wang *et al.*, 2000), metamorphosis (Damjanovski *et al.*, 2000; Ishizuya-Oka, 1996), endocrine-dependent tissue atrophy (Damjanovski *et al.*, 2000), normal tissue turnover (Nakashima *et al.*, 1998; Tessitore *et al.*, 1989), removal of autoimmune T-cells, and killing of cytotoxic T-cells (Deng & Podack, 1993; Nakamura *et al.*, 1996) and NK-cells (Eischen *et al.*, 1996). In thymic tissues, apoptosis of immature CD4+ CD8+ T cells induces positive or negative selections of T-lymphocytes and thus plays an important role in the development of the immune system (McConkey *et al.*, 1994;
Ozeki et al., 1997). On the other hand, in the host–parasite relationship, apoptosis is a natural event by which host animals remove damaged or unnecessary cells and may also be triggered by external stimuli. Bacterial components such as Gram-negative lipopolysaccharide (LPS) (Lakics & Vogel, 1998; Zhang et al., 1993), mycobacterial cord factor (Ozeki et al., 1997), and enterohaemorrhagic Shiga toxin (StX-1 and -2) (Iwata et al., 2002) induce apoptosis, thus demonstrating roles as virulence factor for host animals.

The existence of ceramide or sphingolipid as a major component in bacteria is unique and they may well play a role as a virulence factor. However, it is rather difficult to define whether bacterial ceramide and sphingolipids are a pathological or beneficial component. Kawasaki et al. (1994) reported that in Sphingomonas sp. sphingoglycolipids were a major membrane component, and that these molecules play an important role as a substitute for LPS. In S. spiritivorum, about 20% of the crude lipid consists of sphingolipids, and LPS may be replaced completely or partially. In Gram-negative bacteria, LPS is contained in the outer-membrane bilayer, whilst in Sphingobacterium, sphingolipids are contained in the inner-membrane bilayer. Therefore, it was considered that the sphingolipid of Sphingobacterium could not contact host cells directly and did not play a role in induction of apoptosis. In this study, when HL-60 cells were exposed to live or heat-inactivated S. spiritivorum, apoptosis did not occur, whereas when HL-60 cells were exposed to live Escherichia coli, apoptosis did occur (data not shown). On the other hand, crude lipids of S. spiritivorum, bacterial ceramides and SPLs were found to induce apoptosis. In Gram-negative bacteria, LPS is regarded as the characteristic and essential component which confers potent apoptosis-inducing activity.

In mammalian systems, ceramide plays important roles in the intracellular signal transduction system (Hannun & Obeid, 1995). However, it is difficult to demonstrate the function of ceramide exogenously in vitro because eukaryote-derived ceramides are usually difficult to dissolve in water and do not permeate membranes freely. To conquer this problem, analytical studies on ceramide function have been carried out by sphingomyelinase stimulation or exogenous C2 (acetyl) ceramide administration in vitro (Okazaki et al., 1989, 1990; Schutze et al., 1992). Synthetic C2 or C6 ceramide can induce apoptosis when administered exogenously and is used experimentally for intracellular second messenger analysis in HL-60 cells (Mansat et al., 1997; Okazaki, 1999; Okazaki & Domae, 1994). Mammalian ceramides have a straight-chain fatty acid, while bacterial

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**Fig. 10.** Time-course of caspase activity induced by ceramides and SPLs. HL-60 cells (5 x 10⁵ cells ml⁻¹ in a 35 mm plastic dish) were treated with 500 nM ceramide or SPL for various times. □, Control; ○, cer-A; ●, cer-B; Δ, cerPE-1; ▲, cerPE-2; ▼, cerPI-1; ▽, cerPI-2; ■, cerPM-1. Cytoplasmic extracts were examined for protease activity. Caspase-1 and caspase-3 enzyme activity is expressed as AMC release from fluorogenic substrates. Data represent the mean ± SD of nine experiments. *, P < 0.001 versus control.

**Fig. 11.** Effect of protease inhibitor on apoptosis induced by ceramides and SPLs. HL-60 cells (5 x 10⁵ cells ml⁻¹ in a 35 mm plastic dish) were pre-incubated for 2 h with medium containing 250 µM caspase-1 inhibitor or caspase-3 inhibitor, and treated with 500 nM ceramide or SPL in the presence of inhibitors for 4 h. Cells, prepared as described in Methods, were used to analyse the cell cycle by flow cytometry. Data represent the mean ± SD of nine experiments. *, P < 0.001 versus no inhibitor.
ceramides have a branched-chain fatty acid. In the context of molecular structure and water solubility, branched-chain fatty acids are more soluble than straight-chain fatty acids for molecules with the same number of carbon atoms. It is considered that since bacterial ceramide is more soluble and permeable than mammalian ceramide for HL-60 cell membranes, the bacterial ceramides may be more potent for apoptosis induction. When bacterial ceramide was added to HL-60 cells exogenously, the ceramide content of the cells was significantly increased (Karasavvas et al., 1996), indicating that ceramide is taken into the cell. In the present study, we have shown that for two types of free bacterial ceramides, cer-A (which possesses a non-hydroxy fatty acid) showed greater activity for induction of apoptosis than cer-B (which possesses a non-hydroxy fatty acid). However, almost equivalent activities were observed in the cases of SPLs derived from S. spiritivorum, indicating that the presence of a 2-hydroxy fatty acid in the ceramide moiety seems not to be crucial for inducing apoptosis in vitro. Moreover free fatty acid with an iso-C15 chain did not show any apoptosis-inducing activity, whilst iso-C17 sphinganine from the ceramide showed a weak but significant apoptosis-inducing activity after 2 h incubation in vitro. From these results, taken together with other reports, it was concluded that the minimum ceramide structure necessary for apoptosis induction in HL-60 cells is one possessing isoheptadecasphinganine and non-hydroxy- or 2-hydroxyisopentadecanoic acid.

Ceramide is produced by the action of sphingomyelinase and is thought to be involved in mediating effects of various cytokines (tumour necrosis factor-α, interleukin-1β, interferon-γ), neuronal growth factor, Fas ligand, and ionizing radiation (Saba et al., 1996). Some reports have indicated that ceramide is a primary signalling molecule in Fas-induced cell death (Tepper et al., 1995). To clarify the mechanism of exogenous ceramide-induced apoptosis in HL-60 cells, we examined the possible involvement of Fas surface antigen. Fas is already known to be a type 1 membrane protein and to be activated by binding of the Fas ligand or an antagonistic anti-Fas antibody in Fas-bearing cells (Ferrarini et al., 1999; Schlegel et al., 1996). Fas activation results in caspase activation, which is an apoptosis-inducing signal (Suzuki et al., 1999). DNA endonuclease activation is stimulated as part of the apoptotic process and causes fragmentation of DNA (Mizushima et al., 1996), condensation of nuclei and cytoplasm, convolution of plasma membranes and nuclear or cellular fragmentation. In our study, bacterial ceramides were found to induce Fas expression 2–4 h after stimulation with cer-A or cer-B. Activation of caspase-3, but not caspase-1, by bacterial ceramide administration was maximal at 1 h after stimulation and then gradually decreased. Also, marked DNA fragmentation was observed even at 1 h after stimulation. These results suggest that at least two (and possibly more) mechanisms are involved in apoptosis, both mediated by ceramide derived from S. spiritivorum: a Fas-independent mechanism at an early stage of stimulation and a Fas-dependent mechanism at a later stage. However, direct evidence for ceramide-mediated binding of Fas-ligand to Fas remains to be demonstrated.

Bacterial SPLs induced Fas expression at 2–4 h after stimulation with cerPE-1, cerPE-2, cerPl-1, cerPl-2 or cerPM-1. Activation of caspase-3, but not caspase-1, by these five SPLs was maximal at 3 or 4 h after stimulation. However, marked DNA fragmentation was observed as early as 1 or 2 h after stimulation. These results suggest that a caspase-3-independent mechanism is involved in the SPL-induced apoptosis at an early stage of stimulation and a caspase-3-dependent mechanism at a later stage. The data also suggest that there is a difference in the response of HL-60 cells to bacterial ceramides and SPLs.

Some targets are known to be the downstream signal of ceramide. From this study, it is suggested that bacterial ceramides activated signalling molecules via caspase cleavage, and then induced apoptosis. However, we have not yet studied the details of the phosphorylation–dephosphorylation system involved in signalling. Such analysis will allow clarification of the versatility of ceramide signalling in cells, with regard to chronological and spatial regulation and cross-talk with other lipid signalling. Further detailed biochemical or immunological studies are necessary to clarify the function of bacterial sphingolipids in the host animals.

REFERENCES


