Induction of apoptotic DNA fragmentation and cell death by natural ceramide

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Abstract We have found that a solvent mixture of ethanol and dodecane was able to disperse natural ceramide (Cer) into aqueous solution. In U937 cells, addition of natural Cer to medium caused a characteristic pattern of DNA fragmentation, which is indistinguishable from that caused by TNFα. The effective concentration of Cer is as low as 25 nM. The present study provides direct evidence that natural Cer functions as a second messenger mediating TNFα-induced DNA fragmentation. The use of this solvent to deliver hydrophobic natural Cer to cells will contribute to the elucidation of the biological function of Cer.

Key words: Pregrammed DNA fragmentation; Apoptosis; TNFα; Ceramide; Sphingomyelinase

1. Introduction

Ceramide (Cer) of mammalian tissues is composed of a long-chain base and amide-linked fatty acids that have acyl chain lengths of 16–24 carbon atoms. Involvement of Cer in the transduction pathway of TNFα signal is suggested by the evidence that exogenous bacterial SMase and synthetic C_2- or C_8-Cer are able to mimic the effect of TNFα in the induction of programmed cell death [1,2].

Due to insolubility of natural Cer in water, manipulation of intracellular Cer level is performed through delivery of chemically synthesized Cer with fatty acids of 2–8 carbon atoms or exogenous addition of bacterial SMase. Since the TNFα-induced SM hydrolysis leads not only to Cer production but also the decrease of SM levels, the use of exogenous SMase cannot answer whether the decreased SM level or the produced Cer mediates the function of TNFα. Also, possible effect of molecular heterogeneity of Cer moiety on its biological exertion has never been considered. Though synthetic Cer has been successfully shown to mimic the effect of TNFα in literature, one cannot exclude a possibility that the shortening of fatty acid chain length may lower the affinity of Cer to its targets or cause non-physiological or artificial effect. In the present study, a solvent system was developed to deliver natural Cer to cells in culture. Since DNA fragmentation is the most characteristic biochemical marker for programmed cell death [3–5], the ability of natural Cer in induction of DNA fragmentation was examined. We have shown here that the natural Cer is capable of inducing a characteristic pattern of endonucleolytic DNA cleavage in U937 cells.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium; SM, sphingomyelin; Cer, ceramide; Sph, sphingosine; SMase, sphingomyelinase; TNFα, tumor necrosis factor α; C_2-Cer, *N*-acetylsphingosine; DAG, diacylglycerol; C_6-NBD-Cer, *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-erythro-sphingosine; PMA, 4β-phorbo 12-myristate 13-acetate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

2. Materials and methods

2.1. Materials

[^{14}C]Palmitic acid (spec. act. 45 mCi/mmol) were purchased from NEN, recombinant SMase (10 units/mg protein) from Funakoshi, Japan. Cer from bovine brain SM, Cer from bovine brain cerebroside, DAG and sphingosine were purchased from Sigma. HPTLC plates (silica gel 60) were from Merck, Germany, dodecane from Nacalai tesque, Japan, EDC from PIERCE, recombinant TNFα from Boehringer-Mannheim Biochemica, Germany.

2.2. Cells and culture conditions

U937 human promonocytic cells were grown in an equal volume mixture of Dulbeco's modified Eagle's medium and Ham's F12 medium (DME/F12) supplemented with 10% heat-inactivated fetal calf serum, 100 U penicillin/ml and 0.1 mg streptomycin/ml. Treatment of cells with TNFα and SMase was carried out in serum-free DME/F12 medium supplemented with 10 μg/ml insulin and 10 μg/ml transferrin.

2.3. Addition of natural Cer

Cer (from bovine brain SM or cerebroside), C_2-Cer, DAG, or Sph was first dissolved in ethanol:dodecane (98:2 v/v). The solution was added to above serum-free medium in a tube, and mixed well with the medium by agitation. Final concentrations of ethanol and dodecane were 0.49% and 0.01%, respectively, in all of experiments. Then, the medium was transferred to culture dishes, followed by dispersion of cells into the medium with gentle shaking.

2.4. DNA isolation and gel electrophoresis

DNA was prepared using standard method [6]. Cells were washed with cold phosphate-buffered saline, and the cell pellet was resuspended in 200 μl of a solution containing 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% sodium dodecyl sulfate, and 0.1 mg/ml proteinase K. Proteolytic digestion was allowed to proceed at 50°C for 12–16 h. The sample was extracted with phenol/chloroform/isoamyl alcohol and DNA was precipitated with 1 vol. of 7.5 M NH_4Ac and 2 vols. of absolute ethanol, resuspended in 10 mM Tris-HCl/1 mM EDTA/0.1% SDS buffer, pH 8.0 and treated with 1 μg/ml DNase-free RNase at 37°C overnight, followed by organic extraction and ethanol precipitation, as above, prior to loading onto a 2% agarose gel containing 0.1 mg/ml ethidium bromide. DNA was visualized under UV light and photographed.

2.5. Synthesis of [^{14}C]palmitoylSph and lipid analysis

[^{14}C]Palmitoyl-Sph was synthesized by the method described by Hammarström S. et al. [7] and purified by silicic acid column as described [8]. For determination of Cer incorporation into cells, [^{14}C]palmitoyl-Sph was delivered as described above. After different period of incubation at 37°C, cells were harvested by centrifugation and washed twice with DME/F12 medium containing 10% FCS at room

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temperature. Lipids were extracted by chloroform:methanol (2:1 v/v). An aliquot was counted by a scintillation counter for determination of Cer that was incorporated into cells. Cer was separated on HPTLC plate by a developing solvent, chloroform:methanol:acetic acid (94:1:5 v/v), and other lipids by a second development with chloroform:methanol:water (60:35:8 v/v). Each radioactive lipid separated on HPTLC plate was analyzed and quantitated by image analyzer, BAS 2000 (Fuji Film, Tokyo).

3. Results and discussion

3.1. Incorporation of natural Cer into U937 cells

Most of amphiphilic lipids can be solubilized in aqueous solution when they are dispersed into aqueous solution in ethanol. However, Cer cannot be dispersed into aqueous solution from ethanol stock solution because of the highly hydrophobic long-chain hydrocarbon tail of Cer. In fact, ethanol alone was not effective to deliver [^{14}C]Cer to cells (Fig. 1A).

We found that a mixture of ethanol and dodecane could quickly solubilize Cer and help the dispersion of Cer into aqueous solution. An aqueous solution of 1 ml to which 5 μl of 0.1 mM Cer in ethanol:dodecane (98:2 v/v) was dispersed to form a final concentration of 0.5 μM Cer, 0.49% ethanol and 0.01% dodecane, respectively, looked slightly milky white as does a liposome suspension. Among alkane derivatives examined, decane and undecane have also similar effects. Examination of the Cer solution by laser light scattering showed that the average diameter of the lipid particles made up of ethanol/dodecane/Cer was 350–400 nm, with cell surface z-potential of −22.9 to −10.1 mV. (Details will be described elsewhere.) Without Cer, no lipid particle was detected by laser light scattering.

To examine whether this vehicle can deliver the Cer into cells, we have studied the cellular uptake of [^{14}C]Cer labeled at the fatty acid moiety by [^{14}C]palmitic acid. The uptake of the radioactive Cer appeared as two phases (Fig. 1A). At the initial phase (within 40 min), cellular radioactivity increased rapidly, followed by a slower phase of incorporation. Prompt conversion of incorporated Cer into SM was observed (Fig. 1A and B). The level of incorporated Cer reached a maximum after 1 h, thereafter, gradually decreased probably due to its conversion to SM and degradation of Cer (Fig. 1A and B). These kinetics of Cer incorporation are consistent with that reported in the literature where the uptake of Cer by cells has been studied in detail by using a fluorescent Cer analogue, C_6-NBD-Cer, which is found to be able to insert into plasma membrane through the hydrophobic tail, even at 2°C [9].

3.2. DNA fragmentation induced by natural Cer

TNFα has been demonstrated to cause DNA fragmentation in U937 cells (Fig. 2A) [1,10–12]. TNFα has been reported to cause an early hydrolysis of SM in U937 cells [1]. If SM or its derivatives such as Cer or Sph mediate the action of TNFα, the most important criteria are whether the artificial manipulation of the level of those materials can trigger the same responses as that induced by TNFα and whether those responses occur within a period comparable to that required for responses to TNFα action. A recombinant SMase from *Bacillus cereus* was used for the hydrolysis of SM on the surface of U937 cells. Treatment of cells with this SMase caused DNA fragmentation within 2 h (Fig. 2B), which approaches the minimum time required for TNFα and is similar to that for the action of a

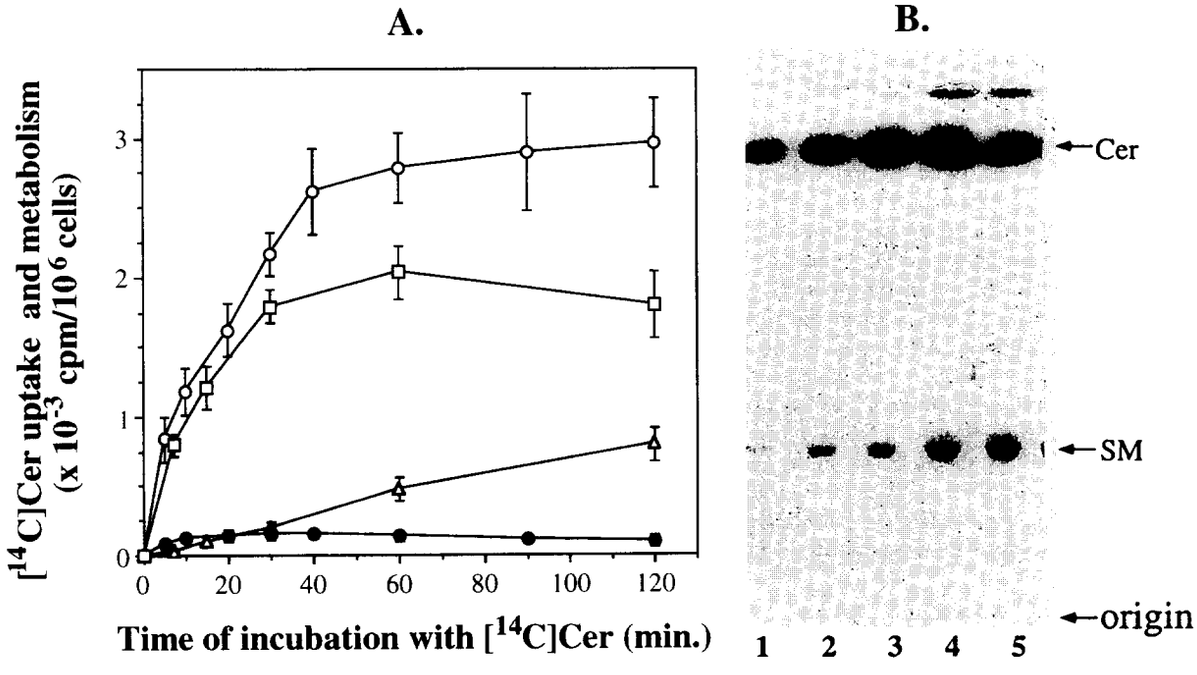

Fig. 1. Uptake and metabolism of exogenous Cer. U937 cells (2×10^6 cells/ml) were incubated at 37°C for the indicated time periods with 500 nM [^{14}C]Cer delivered with ethanol/dodecane (98:2 v/v) (open symbol) or ethanol (closed symbol). Lipids were analyzed as described in section 2. The quantitative data was shown in (A) and an autoradiography pattern of incorporated lipids in (B). In A, each value is the mean ± standard deviation from three independent experiments (n = 6). ○, Total lipid-bound radioactivity incorporated with ethanol/dodecane; ●, total lipid-bound radioactivity incorporated with ethanol; □, Cer-linked radioactivity in cells; △, SM derived from incorporated Cer. Lanes 1–5 in B, labeled products after incubation of 7.5, 15, 30, 60 and 120 min, respectively.

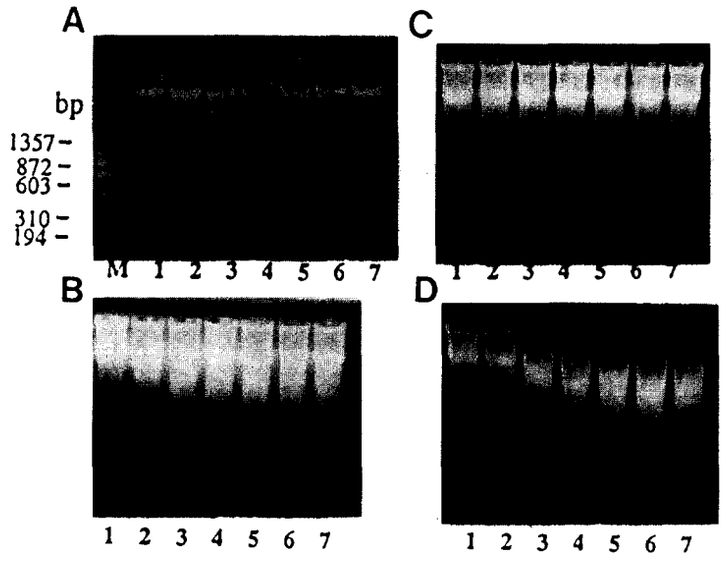

Fig. 2. Time course of DNA fragmentation induced by TNFα, SMase and Cer. U937 cells (2×10^5 cells/ml) were treated with 500 IU/ml TNFα (A), 1 mU/ml SMase (B), ethanol/dodecane only (C) and 200 nM Cer delivered with ethanol/dodecane (D) for the indicated time periods. DNA was prepared and analyzed as described in section 2. In A and B, lanes 1 to 7 represent 0, 1, 2, 3, 4, 5 and 6 h of incubation time, respectively. In C and D, lanes 1 to 7 represent 0, 1, 1.5, 2, 3, 4 and 5 h of incubation, respectively.

SMase purified from *Staphylococcus aureus* (Sigma) [2]. The effect could be detected at a concentration of SMase as low as 0.04 mU/ml (Fig. 3A).

Since hydrolysis of SM by exogenous SMase or TNFα-activated SMase not only reduces the content of SM but also produces degradation products such as Cer and phosphocholine, which may act as messenger system for the transduction of TNFα signals, we tested this possibility by delivering natural Cer with ethanol/dodecane vehicle to U937 cells. Addition of ethanol/dodecane alone did not show any effect on cell morphology, viability and DNA fragmentation (Figs. 2C and 4A). Treatment of U937 cells with natural Cer resulted in shrinkage of cell size and condensed area in nucleus (Fig. 4B). Agarose gel electrophoresis showed the cleavage of DNA into multiples of nucleosome size fragments (Fig. 2D), the profile of which was indistinguishable from that caused by the TNFα or exogenous SMase treatment. The DNA fragmentation profile was detectable after 1.5 h, and evident after 2 h treatment. The effect of natural Cer on DNA fragmentation can be detected at a concentration as low as 25 nM, and was evident at 100 nM (Fig. 3B). C_2-Cer, which was also delivered to U937 cells by ethanol/dodecane vehicle, was effective in inducing DNA fragmentation at concentrations higher than 1 μM (Fig. 3C). This dose-dependence of C_2-Cer is consistent with that reported by Obeid [1], where C_2-Cer was delivered to U937 cells with ethanol. This difference between the potency of natural Cer and C_2-Cer may be attributed to the presence of long-chain fatty acid as compared to C_2-Cer. The specificity of natural Cer was further examined by testing several Cer analogs delivered to cells by ethanol/dodecane. Sph and DAG were ineffective in inducing DNA fragmentation. Interestingly, α-hydroxylation of fatty acid (Cer derived from bovine brain cerebroside contains α-hydroxyl fatty acid) significantly diminished the potency of Cer in inducing DNA fragmentation (data not shown). DNA fragmentation induced by natural Cer as well as TNFα and C_2-Cer was inhibited by Zn^{2+} and PMA (data not shown), suggesting that TNFα and Cer may activate a common final pathway to cause DNA fragmentation and the inhibition of the effect of TNFα by PMA is not only restricted to the regulation of TNFα receptors as reported by Aggarwal and

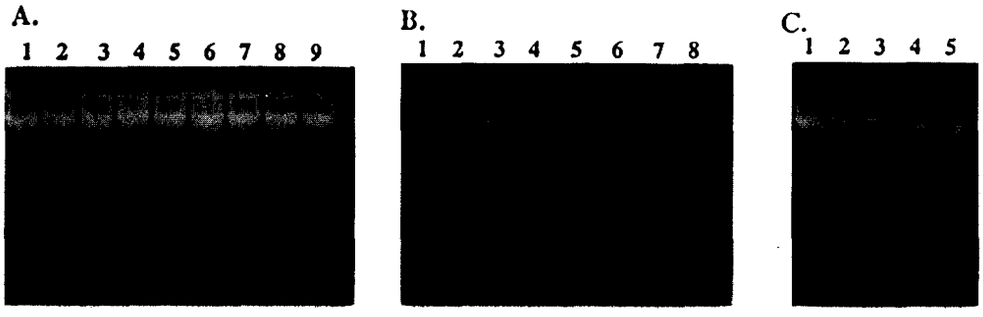

Fig. 3. Effects of concentrations of SMase and Cer on DNA fragmentation. U937 cells (2×10^5 cells/ml) treated with different concentrations of SMase (A) and Cer (B) and C_2-Cer (C) for 3 h. DNA was prepared and analyzed as described in section 2. In A, lanes 1 to 9 represent 0, 0.002, 0.008, 0.04, 0.2, 1, 5, 25 and 125 mU/ml. In B, lanes 1 to 8 represent 0, 6.20, 12.5, 25, 50, 100, 200 and 500 nM. Lanes 1–5 in C are 0, 0.5, 1.0, 3.0 and 5 μM, respectively.

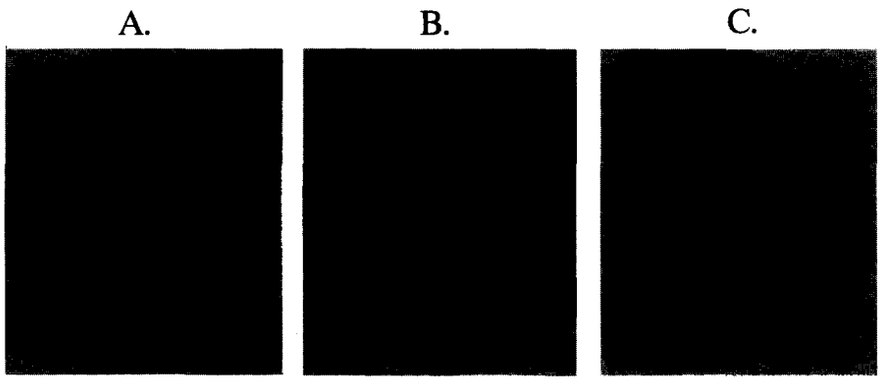

Fig. 4. Changes of cell morphology after Cer treatment. U937 cells (2×10^5 cells/ml) were treated with ethanol/dodecane (A) and Cer (0.2 μM) delivered with ethanol/dodecane (B), as described in section 2. (C) Control cells (without treatment).

Eessalu [13], but is also due to blockage of later signal transduction.

In conclusion, the delivery of natural Cer to U937 cells can induce programmed DNA fragmentation, similar to that induced by TNFα. This Cer-delivering solvent may provide the valuable tools to further explore the biological roles of Cer and their action mechanisms.

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