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Apoptotic Activities of C2-Ceramide and C2-Dihydroceramide Homologues Against HL-60 Cells

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Abstract—The apoptotic activities of non-natural ceramide homologues, C2-*homo*-ceramide, C2-*homo*-dihydroceramide, C2-*bis-homo*-ceramide and C2-*bishomo*-dihydroceramide, were examined using human leukemia HL-60 cells. The apoptotic activity was in order of C2-ceramide > C2-*homo*-ceramide≈C2-*bishomo*-ceramide and the activities of the L-*erythro*- and D-*erythro*-ceramide homologues were similar. The morphological features of the cells, DNA fragmentations, proteolytic processing of pro-caspase-3 and the cleavage of PARP as the result of treatments with these homologues indicated that cell death was induced by apoptosis.

Introduction

Sphingolipids, a diverse class of lipids, have recently been identified as intra- and intercellular signal molecules, which may mediate a number of the cell functions, including cell growth, differentiation, cell–cell contact and apoptosis (programmed cell death).[1] Specifically, several exogenous stimuli, including tumor necrosis factor α, growth factor withdrawal, Fas ligand, anticancer drugs, oxidative stress, heat shock, ionizing radiation and ultraviolet light, all cause an increase in intracellular levels of ceramide via the activation of sphingomyelinase, and induce apoptosis.[2]

Consequently, in many studies, cell-permeable ceramides such as *N*-acetyl sphingosine (C2-Ceramide, C2-Cer) have been used as a very powerful tool to investigate the role of the sphingomyelin hydrolysis reaction in several of the exogenous stimuli. It has recently been reported that C2-Cer treatment activates a family of asparate-specific cysteine proteases, called caspases, which are intimately associated with apoptosis and which cleave a number of substrates such as poly(ADP-ribose) polymerase (PARP).[3]

Chemically prepared ceramide analogues have also been employed to clarify the structural determinants required for the biological action of a ceramide.[4] Aspects of the stereospecificity were investigated using stereoisomers of C2-ceramide and C2-dihydroceramide. The activity of four stereoisomers for cell growth inhibition is in the order L-*threo*-C2-Cer > D-*erythro*-C2-Cer = L-*erythro*-C2-Cer > D-*threo*-C2-Cer in HL-60 human leukemia cells. In addition, DL-*erythro*-C2-dihydroCer was inactive, but DL-*threo*-C2-dihydroCer was active.[4f] The *trans* configuration may not be importance for inducing apoptosis. Both the *cis* isomer and acetylene type derivative of ceramide were more active than the *trans* isomer in human leukemia U937 cells.[4c] Furthermore, synthetic C6-phytoceramide (N-hexanoyl-phytosphingosine) induced higher levels of apoptosis than C6-Cer in SK-N-BE(2)C catecholaminergic neuroblastoma cells.[4g]

As described above, the role of the double bond, secondary hydroxyl group, amino group and long hydrocarbon chain of the ceramide in term of the induction of apoptotic cell death has been examined. However the issue of whether the environment around the primary hydroxyl group is important for the apoptotic activity has not been examined, although Jonghe et al. reported the synthesis of *homo*-ceramides, having a heptyl and a phenyl side chains, and their lack of effect on the axonal growth of hippocampal neurons.[5] To address this problem, we recently introduced methylene spacers

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between primary hydroxyl and amino group of the sphingosine backbone by chemical synthesis, and examined the apoptotic activity of those novel ceramide homologues (namely C2-Cer-1, C2-Cer-2) in cultured human cancer cells.[6] Surprisingly, these homologues, even those containing a two methylene spacer, strongly induced cell death. This indicates that the distance between primary hydroxyl and amino groups is not critical for the cytotoxic activity of a ceramide.

In this paper, we report on a detailed examination at the requirement of the double bond and the stereospecificity of some novel ceramide homologues, C2-Cer-1 and C2-Cer-2, in apoptotic cell death against human leukemia cells using a synthetic cell-permeable ceramide, prepared in our laboratory. This novel ceramide homologue-induced cell death occurs via the well characterized apoptotic pathway. These results provide useful information on structure–function relationships of ceramide-mediated apoptosis, and will be useful in the design of new anti-cancer drugs based on the ceramide structure.

Results and Discussion

Syntheses of ceramide and dihydroceramide homologues

The structures of the compounds used in this study can be classified into three categories; Cer class, Cer-1 class and Cer-2 class as shown in Figure 1. The Cer class includes C2-ceramide and C2-dihydroceramide and their enantiomers. The Cer-1 class includes 3-acylamino-5-nanodecen-1,4-diol and its dihydro-derivatives, having one methylene spacer between the primary hydroxyl and the amino groups of the sphingosine backbone and the Cer-2 class includes 4-acylamino-6-eicosen-1,5-diol derivatives, having two methylene spacers between the primary hydroxyl and amino groups.

D-*erythro*-C2-Ceramide (D-*e*-C2-Cer),[7] D-*erythro*-C2-*homo*-ceramide (D-*e*-C2-Cer-1),[6] D-*erythro*-C2-*bishomo*-ceramide (D-*e*-C2-Cer-2)[8] and their respective enantiomers and dihydro-compounds were synthesized from L- and D-serine, aspartic acid and glutamic acid, respectively. L-*t*-C2-DHCer was synthesized as described by Azuma et al.[7] L-*t*-C2-DHCer-1 and its enantiomer D-*t*-C2-DHCer-1 were isolated from their respective minor diastereomers, which was separated by silica gel chromatography in similar manner to that described previously.[6]

Structure–function relationships of C2-ceramide homologues

To clarify the molecular mechanism involved in cytotoxicity, we analyzed the effects of C2-ceramide homologues on the apoptosis of HL-60 cells. Cell death was determined by treatment with 10 μM ceramide homologues against HL-60 cells after 6 h of stimulation using the MTT assay.[9] C2-Cer and C2-DHCer were used as a positive and a negative control, respectively. The percent of apoptosis for the Cer-1 class and their activities are shown in Figure 2A, and are in the order L-*e*-C2-Cer≈D-*e*-C2-Cer > L-*e*-C2-Cer-1≈D-*e*-C2-Cer-1 > D-*t*-C2-DHCer-1≈L-*t*-C2-DHCer-1 > L-*t*-C2-DHCer > L-*e*-C2-DHCer-1≈D-*e*-C2-DHCer-1≈D-*e*-C2-DHCer, and 2B indicates the activities of the Cer-2 class, which are in the order D-*e*-C2-Cer-2≈L-*e*-C2-Cer-2 > L-*e*-C2-DHCer-2≈D-*e*-C2-DHCer-2.

D-*e*-C2-Cer-1 and L-*e*-C2-Cer-1 indicated low but significant apoptotic activities in comparison with D-*e*-C2-Cer and L-*e*-C2-Cer, and that the activities were comparable to those of D-*e*-C2-Cer-2 and L-*e*-C2-Cer-2.

When the overall apoptotic activity is compared, the order is C2-ceramide > C2-*homo*-ceramide≈C2-*bishomo*-ceramide,[6] with the L-*erythro*- and the D-*erythro*-ceramide

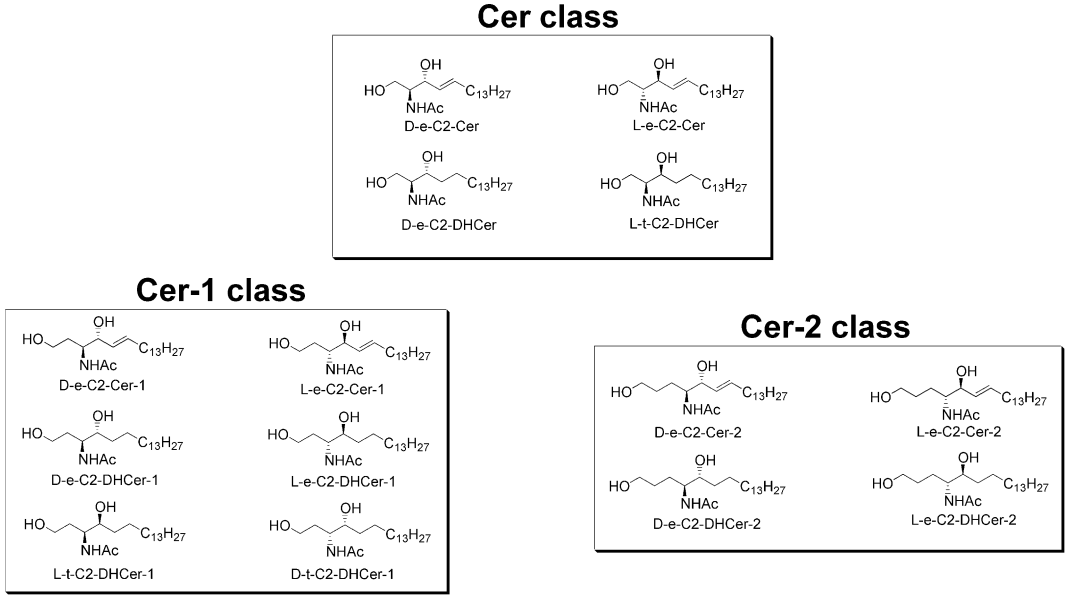

Figure 1. Chemical structures of the three classes of ceramide homologues.

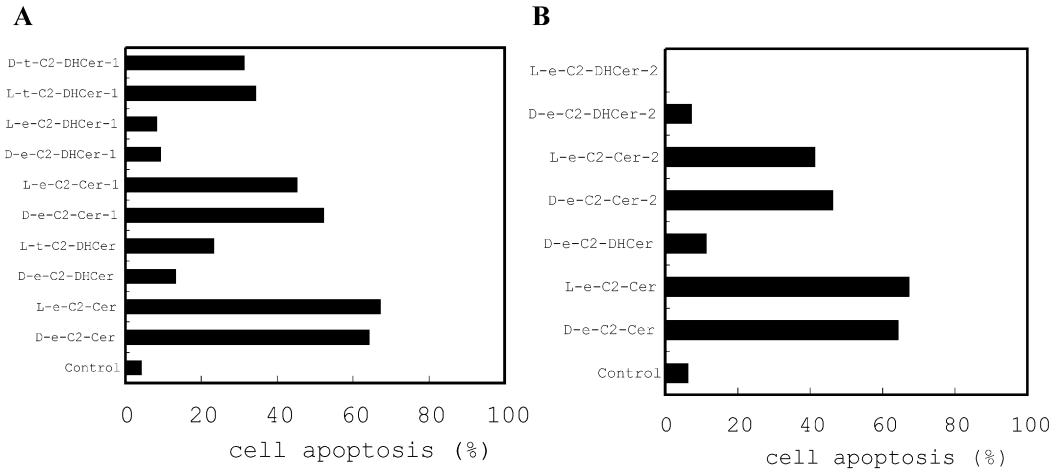

Figure 2. Percent of cell apoptosis after a 6 h treatment with 10 μM ceramide homologues in HL-60 cells. Values are the average of at least three separate experiments. **A** Cer-1 class, **B** Cer-2 class.

homologues showing about the same apoptotic activities. Therefore, these results show that the optical isomerism and the location of the primary hydroxyl group in C2-ceramide homologues has no significant effect on their apoptotic activities. Bielawska et al.[4f] and Chang et al.[4a] reported that the cell toxicity of D-*e*-C2-Cer is similar to L-*e*-C2-Cer.

The morphological feature of HL-60 cells after incubation with 10 μM ceramide homologues for 6 h is shown in Figure 3. In a previous study,[3] D-*e*-C2-Cer are found to induce the condensation of chromatin with a reduction in nuclear size, which are characteristic of apoptosis. The morphological feature was observed in HL-60 cells, after treatment with D-*e*-C2-Cer (b), L-*e*-C2-Cer, D-*e*-C2-Cer-1 (c), L-*e*-C2-Cer-1, D-*e*-C2-Cer-2 (d) and L-*e*-C2-Cer-2.

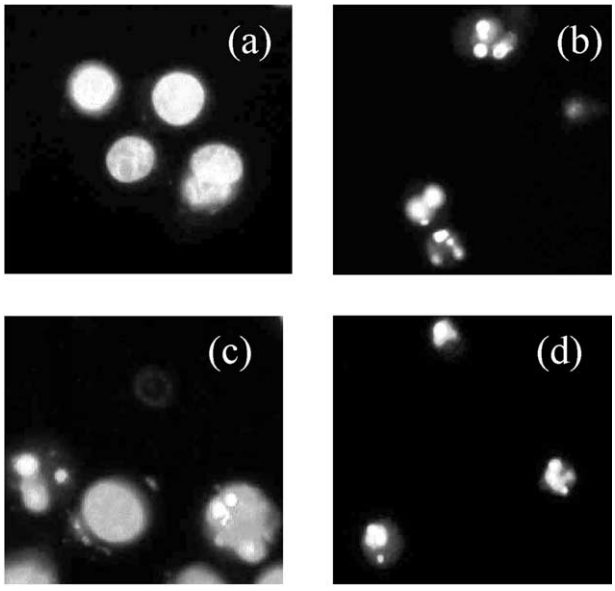

Figure 3. Morphological features of HL-60 cells after treatment with (a) ethanol vehicle; (b) D-*e*-C2-Cer; (c) D-*e*-C2-Cer-1; (d) D-*e*-C2-Cer-2 after 6 h.

DNA fragmentation by the ceramide homologues were observed with 10 μM after 8 h. As shown in Figure 4, a large quantity of DNA fragmentations were induced when stimulated with D-*e*-C2-Cer (lane b), L-*e*-C2-Cer (c), D-*e*-C2-Cer-1 (f), and L-*e*-C2-Cer-1 (g) in Figure 4A and D-*e*-C2-Cer-2 (e) and L-*e*-C2-Cer-2 (f) in Figure 4B. On the other hand, the respective dihydro-ceramide homologues in Figure 4 resulted in a small quantity of DNA fragmentation. A good relation between cell death, morphological changes and DNA fragmentation by these ceramide homologues, was found.

To elucidate the involvement of caspase, which is associated with apoptosis, the activation of caspase-3 was analyzed in HL-60 cells. The proteolytic processing of pro-caspase-3 in response to exogenous ceramide homologues was examined by Western blotting using a polyclonal anti caspase-3 antibody. This caspase is synthesized as a 32 kDa precursor which, after cleavage, results in the 17 kDa subunit (the active form).[10] Exogenous C2-ceramide is known to induce the processing and activation of caspase-3 in cells.[3] Cells treated with not only C2-ceramide but also *homo*- and *bishomo*-ceramide, generated the active form, as shown in Figure 5A [D-*e*-C2-Cer-1 (lane f) and L-*e*-C2-Cer-1 (g)] and in Fig. 5**B** [D-*e*-C2-Cer-2 (e) and D-*e*-C2-Cer-2 (f)].

Ceramide generation has recently been shown to cleave PARP, a known substrate for caspase-3, in TNF-α mediated apoptosis.[11] In addition, in the case of cells treated with exogenous C2-ceramide, the cleaved of PARP is observed.[3] The cleavage of PARP was examined under cell death induced by ceramide homologues. The cleaved form of PARP was observed in HL-60 cells treated with D-*e*-C2-Cer (lane b), L-*e*-C2-Cer (c), D-*e*-C2-Cer-1 (f) and L-*e*-C2-Cer-1 (g) in Figure 5C and with D-*e*-C2-Cer-2 (lane e) and L-*e*-C2-Cer-2 (f) in Figure 5D.

These observations, the morphological features of cells, DNA fragmentation, the proteolytic processing of the pro-caspase-3 and cleavage of PARP, by the treatment with C2-*homo*- and C2-*bishomo*-ceramide clearly

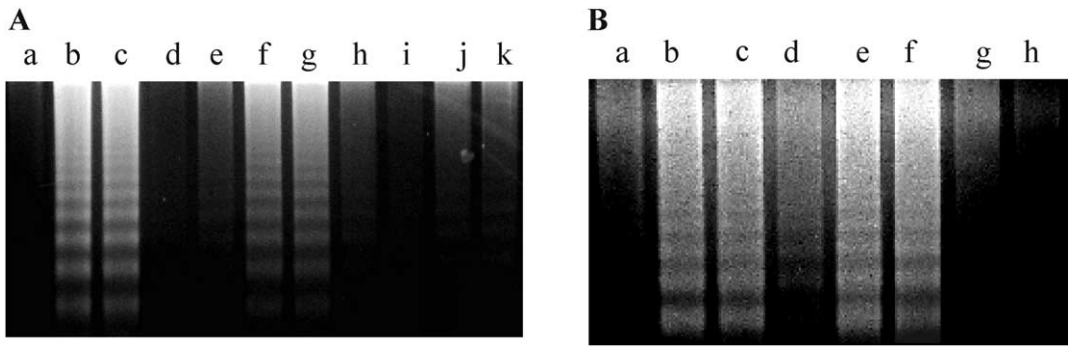

Figure 4. DNA fragmentation of HL-60 cells after treatment with ceramide homologues (10 μM) after 8 h. **A**: (a) ethanol vehicle; (b) D-*e*-C2-Cer; (c) L-*e*-C2-Cer; (d) D-*e*-C2-DHCer; (e) L-*t*-C2-DHCer; (f) D-*e*-C2-Cer-1; (g) L-*e*-C2-Cer-1; (h) D-*e*-C2DHCer-1; (i) L-*e*-C2-DHCer-1; (j) L-*t*-C2-DHCer-1 and (k) D-*t*-C2-DHCer-1. **B**; (a) ethanol vehicle; (b) D-*e*-C2-Cer; (c) L-*e*-C2-Cer; (d) D-*e*-C2-DHCer; (e) D-*e*-C2-Cer-2; (f) L-*e*-C2-Cer-2; (g) D-*e*-C2-DHCer-2 and (h) L-*e*-C2-DHCer-2.

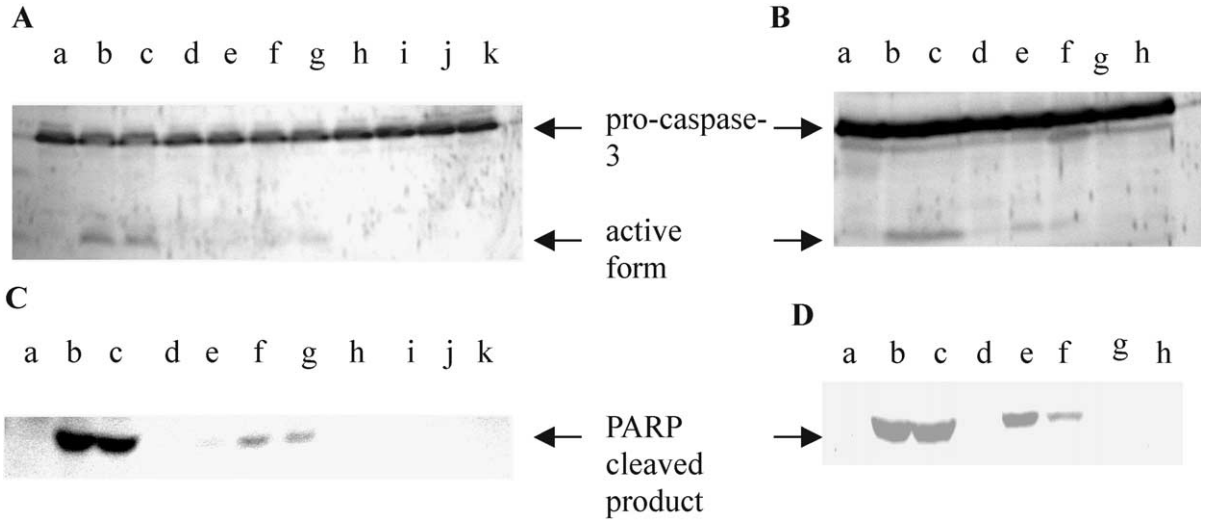

Figure 5. Western blot analysis of caspase-3 (**A**, **B**) and PARP (**C**, **D**) in HL-60 cells with ceramide homologues (10 μM) after 6 h. **A** and **C**; (a) ethanol vehicle; (b) D-*e*-C2-Cer; (c) L-*e*-C2-Cer; (d) D-*e*-C2-DHCer; (e) L-*t*-C2-DHCer; (f) D-*e*-C2-Cer-1; (g) L-*e*-C2-Cer-1; (h) D-*e*-C2-DHCer-1; (i) L-*e*-C2-DHCer-1; (j) L-*t*-C2-DHCer-1 and (k) D-*t*-C2-DHCer-1. **B** and **D**; (a) ethanol vehicle; (b) D-*e*-C2-Cer; (c) L-*e*-C2-Cer; (d) D-*e*-C2-DHCer; (e) D-*e*-C2-Cer-2; (f) L-*e*-C2-Cer-2; (g) D-*e*-C2-DHCer-2 and (h) L-*e*-C2-DHCer-2.

indicate that cell death is induced by apoptosis, as has been reported for ceramide and C2-ceramide.

Time- and dose-dependent HL-60 cell death induced by ceramide homologues

We further investigated the effects of D-*e*-C2-Cer, D-*e*-C2-Cer-1 and D-*e*-C2-Cer-2, which are capable of inducing apoptosis, and D-*e*-C2-DHCer, which is not.

Time- and dose-dependent apoptosis induced by the incubation of HL-60 cells with D-*e*-C2-Cer, D-*e*-C2-Cer-1, D-*e*-C2-Cer-2 and D-*e*-C2-DHCer are shown in Figure 6. The apoptosis of HL-60 cells progressively increases with increasing incubation time and concentrations of D-*e*-C2-Cer, D-*e*-C2-Cer-1, D-*e*-C2-Cer-2, respectively, expect for D-*e*-C2-DHCer. Because of the activated expression of apoptosis, the concentration of C2-*homo*- and *bishomo*-ceramide in excess of 5 μM may be necessary to achieve apoptosis.

The time- and dose-dependent cleavage of PARP and the generation of the proteolytically processed form of caspase-3 were also observed when HL-60 cells were treated with these ceramide homologues. (data not shown)

The direct target of ceramide for intracellular apoptotic signal transduction has not been ascertained, although some candidates, a ceramide-activated kinase,[12] ceramide-activated protein phosphatase (CAPP),[13] PKC ζ[14] and mitochondrial membrane,[15] have been proposed. In the future, novel ceramide homologues, such as those described in this paper, will be useful to reveal whether these factors constitute a target through a biological binding assay or by pore-formation in membranes.

The finding of this study can be summarized as follows:

(1) The observed phenomena, morphological features of cells, DNA fragmentation, proteolytic processing of pro-caspase-3 and the cleavage of PARP, by treatment with C2-*homo*- and C2-*bishomo*-ceramide clearly indicate that cell death is induced by apoptosis as in the case of ceramide and C2-ceramide.

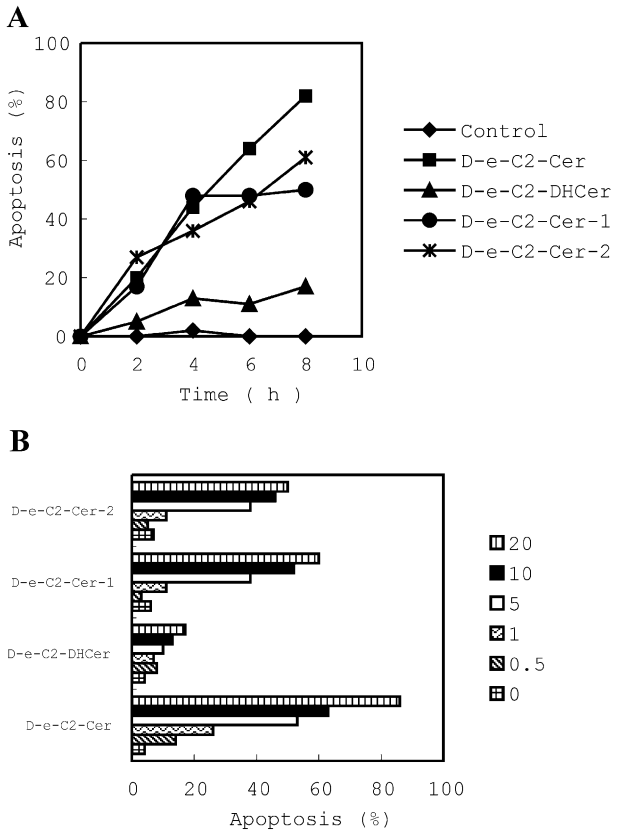

Figure 6. Time- and Dose-dependent HL-60 cell death induced by ceramide homologues. (**A**) Cells were incubated with 10 μM of ceramide homologues. (**B**) Cells were incubated with 0–20 μM concentrations for 6 h and values are the average of at least three separate experiments.

(2) The apoptotic activity was in the order C2-ceramide > C2-*homo*-ceramide≈C2-*bishomo*-ceramide. The location of the primary hydroxyl group on the C2-ceramide homologues has no effect on their apoptotic activities.
(3) L-*erythro*- and D-*erythro*-ceramide homologues showed similar apoptotic activities, therefore these apoptotic activities are not enantiospecific.

The resulting apoptosis found here may have implications in cancer chemotherapy and provide significant information for the creation of new anti-cancer agents.

Experimental

Syntheses of C2-ceramide and dihydroceramide homologues

The compounds used in this study were synthesized according to our previous papers.[6–8]

New compounds

(3*R*, 4*S*, 5*E*)-3-Acetylamino-nonadec-5-en-1,4-diol (L-*e*-C2-Cer-1) HRMS (FAB, positive), calcd for $C_{21}H_{42}NO_3$: $(M+H)^+$ 356.3165; found 356.3176.

(3*R*, 4*S*)-3-Acetylamino-nonadecane-1,4-diol (L-*e*-C2-DHCer-1) HRMS (FAB, positive), calcd for $C_{21}H_{44}NO_3$: $(M+H)^+$ 358.3321; found 358.3335.

(3*S*, 4*S*)-3-Acetylamino-nonadecane-1,4-diol (L-*t*-C2-DHCer-1) HRMS (FAB, positive), calcd for $C_{21}H_{44}NO_3$: $(M+H)^+$ 358.3321; found 358.3313.

(3*R*, 4*R*)-3-Acetylamino-nonadecane-1,4-diol (D-*t*-C2-DHCer-1) HRMS (FAB, positive), calcd for $C_{21}H_{44}NO_3$: $(M+H)^+$ 358.3321; found 358.3336.

(4*R*, 5*S*, 6*E*)-4-Acetylamino-6-eicosen-1,5-diol (L-*e*-C2-Cer-2) HRMS (FAB, positive), calcd for $C_{22}H_{44}NO_3$: $(M+H)^+$ 370.3321; found 370.3346.

(4*R*, 5*S*)-4-Acetylamino-eicosan-1,5-diol (L-*e*-C2-DHCer-2) HRMS (FAB, positive), calcd for $C_{22}H_{46}NO_3$: $(M+H)^+$ 372.3478; found 372.3495.

Cell culture

Human pronyelocytic leukemia HL-60 cells were grown in RPMI 1640 medium (Sigma) containing 5% heat-incubated fetal bovine serum supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin. On the day of the experiment, cells were washed twice in serum-free RPMI 1640 and resuspended in the serum-free medium ($0.4–1\times10^6$ cells/mL). The cells were treated with various ceramide homologues for 6 or 8 h at 37 °C, and an MTT assay was run or DNA fragmentation was observed. All the ceramide homologues were dissolved in ethanol. Control experiments were performed with ethanol (0.1%) as the vehicle.

MTT assay

The apoptosis modulus was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored, water insoluble formazan salt. 4×10^4 cells/100 μL were plated in 96-well dishes. Ceramide homologues were dissolved in ethanol at a stock concentration of 0–20 mM and then diluted with serum free medium. The final concentration of ethanol was 0.1%. 10 μL of 5 mg/mL MTT was added to each well after 4 h. The reaction was stopped by adding 100 μL of 0.04 N HCl in isopropanol after 6 h. The absorbance at a wavelength of 570 nm was measured. Percent apoptosis was calculated as follows: % = (100−living cell %). All results were determined in triplicate.

DNA Fragmentation

HL-60 cells (2×10^6 cells/2 mL) were plated in 6-well dishes. Ceramide homologues were dissolved in ethanol at a stock concentration of 10 mM and then diluted with serum free medium. After an 8 h incubation, the cells were collected by centrifugation at 2500 rpm for 5 min at 4 °C. Cells were lysed in 100 μL of lysis buffer (10 mM Tris–HCl; pH 7.4, 10 mM EDTA; pH8.0, and 0.5% Triton X-100). Soluble cell lyses were collected by

centrifugation at 15,000 rpm for 5 min. Cell lysates were treated for 1 h at 37 °C with RNaseA (0.2 mg/mL). Proteinase K (0.2 mg/mL) was added, and sample was incubated at 50 °C for 30 min. 5 M NaCl (20 μL) and isopropanol (120 μL) were added and the sample was incubated at −20 °C for 12 h. DNA pellets were collected by centrifugation at 15,000 rpm for 15 min, and dissolved in 20 μL of a TE buffer (10 mM Tris–HCl; pH 7.4, 1 mM EDTA; pH8.0). The DNA was then analyzed on a 2% agarose gel. The DNA in the gels was visualized under a UV light after staining with ethidium bromide.

Staining of apoptotic nuclei

HL-60 cells were treated with ceramide homologues as described above. After an 6 h incubation, cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature, and then rinsed with PBS. Thereafter, the cells were stained with the 4′,6-diamidino-2-phenylindole (DAPI) μg/mL in PBS for 30 min at room temperature and rinsed with PBS. Condensed nuclei were detected by Olympus IX-71 fluorescence microscopy.

Immunoblot analysis

HL-60 cells were treated with ceramide homologues as described '*DNA Fragmentation*'. After an 6 h incubation, cells were collected by centrifugation at 4000 rpm for 5 min at 4 °C, washed twice with PBS and lysed in 100 mL of SDS sample buffer (62.5 mM Tris–HCl; pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue, 5% β-mercaptoethanol). Cell lysates were boiled for 3 min and separated on 14% SDS-polyacrylamide gels, transferred to Immobilon-P transfer membrane (Millipore), and probed with rabbit polyclonal anti-caspase-3 antibody (Santa Cruz Biotechnology), followed by goat anti-rabbit antibody coupled to alkaline phosphatase (Sigma).

For PARP immnoblots, cell lysates were separated on 8% SDS-polyacrylamide gels, transferred to Immobilon-P transfer membrane, and probed with monoclonal mouse anti-human PARP (Trevigen), followed by goat anti-mouse antibody coupled to alkaline phosphatase (Sigma).

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