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Notes

Effects of α- and β-Galactosylated C2-Ceramides on the Immune System

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In contrast to the immunosuppressive effects of C2-ceramide (C2-Cer), α-galactosylceramides with ceramides having more than 10 carbons in fatty acid chains have immunostimulatory activities. We therefore synthesized α- and β-galactosylated C2-Cers in order to examine their effects on the immune system. β-Galactosylated C2-Cer and C2-Cer suppressed the allogeneic mixed leukocyte reaction (MLR) responses, but α-galactosylated C2-Cer stimulated the MLR response.

Introduction

Apoptosis, or active cell death, is a process that occurs under a range of physiological and pathological conditions in many different cell types. In leukemia cell lines, C2-ceramide (C2-Cer) induces apoptosis via the sphingomyelin pathway.[1,2] Further study demonstrated that the Ras signaling pathway initiated by C2-Cer mediates Fas-induced apoptosis in leukemia lines.[3] In addition, Olshefski et al. recently suggested that not only C2-Cer but also glycosylated C2-Cer (ganglioside) has immunosuppressive activity based on the fact that C2-Cer GM3 and C2-Cer inhibited the proliferation of lymphocytes.[4] Thus, C2-Cer and its derivatives have drawn much attention as useful tools to investigate the mechanism of action of apoptosis.

We previously reported that the α-galactosylceramides (α-GalCers; galactose binds to ceramide in an α-configuration), which have dihydroceramide portions with more than 10 carbons in fatty acid chains, have potent immunostimulatory and antitumor activity[5] and that β-GalCers, which have the same ceramide moieties as their related α-GalCers, possess little immunostimulatory effects.[6,7] In consideration of both the above findings and our findings, the question arose as to whether α-galactosylated C2-Cer (α-Gal-C2-Cer) and β-galactosylated C2-Cer (β-Gal-C2-Cer) have immunosuppressive or immunostimulatory effects. To study their properties on the immune system, we synthesized α-Gal-C2-Cer and β-Gal-C2-Cer.

In this paper, we describe the syntheses of α-Gal-C2-Cer and β-Gal-C2-Cer and the effects of α-Gal-C2-Cer, β-Gal-C2-Cer, and C2-Cer on allogeneic mixed leukocyte reaction (MLR) responses using murine spleen cells.

Chemistry

The α-Gal-C2-Cer was synthesized from a commercially available C2-Cer as follows (Scheme 1). The C2-Cer (**1**) was directly glycosylated to **3** under conditions using benzyl-protected galactosyl fluoride donor **2** with $AgClO_4$ and $SnCl_2$. Benzyl-protected α-Gal-C2-Cer **3** was treated with sodium in liquid ammonia, and debenzylation proceeded smoothly to give α-Gal-C2-Cer (**4**). The β-Gal-C2-Cer (**5**) was synthesized by the method of Shapiro et al.[8]

Results and Discussion

To study the immunostimulatory or immunosuppresive properties of α-Gal-C2-Cer and β-Gal-C2-Cer, we examined effects of both the compounds and parental C2-Cer on allogeneic MLR using murine spleen cells. As shown in Figure 1, C2-Cer suppressed the MLR response at concentrations from 80 ng/mL to 2 μg/mL in a concentration-dependent manner. β-Gal-C2-Cer also inhibited the MLR response in a concentration-dependent manner at above-mentioned concentrations, and this immunosuppressive potency was weaker than that of C2-Cer. In contrast to these compounds, α-Gal-C2-Cer induced concentration-dependent stimulation of the MLR responses in concentrations from 80 ng/mL to 2 μg/mL.

It is quite interesting that the difference configuration of the glycosidic linkage between galactose and C2-Cer induces contrasting immune responses in murine spleen cells, i.e., α-Gal-C2-Cer and β-Gal-C2-Cer have immunostimulatory and immunosuppressive activities, respectively. This is the first demonstration that α-Gal-C2-Cer and β-Gal-C2-Cer have contrasting effects on the immune system.

Recently it was reported that exogenously added C2-Cer suppresses the antigen-presenting function of dendritic cells (DC).[9] In contrast, we found that KRN7000, an α-GalCer ((2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol), activates the immune system via enhancement of the antigen-presenting function of DC.[10] It will be of interest to examine the effects of α-Gal-C2-Cer, β-Gal-C2-Cer, and C2-Cer on DC.

Taking these findings together, the set consisting of α-Gal-C2-Cer, β-Gal-C2-Cer, and C2-Cer can be considered to be a useful tool to investigate the mechanism of apoptosis and the immune reponses induced by DCs.

Experimental Section

Chemical Methods. Column chromatography was performed on silica gel (Cica-MERCK silica gel 60, particle size

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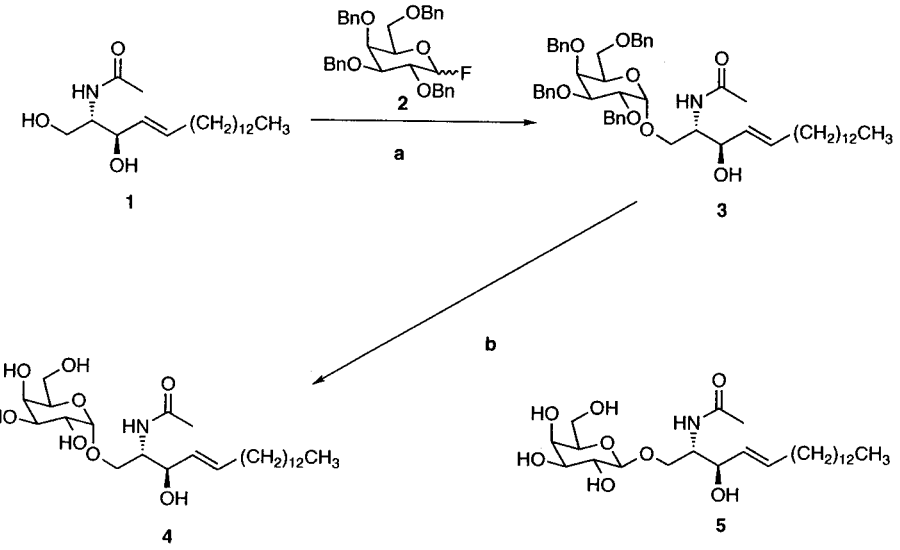
Scheme 1[a]



[a] (a) **2**, $SnCl_2$, $AgClO_4$, MS4A/THF, −10 °C to rt, 2 h; (b) Na/NH_3, 2 h.

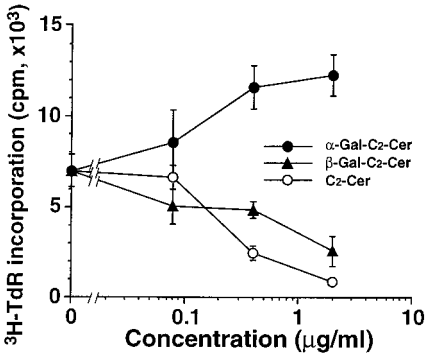


Figure 1. Effects of α-Gal-C2-Cer (**4**), *β*-Gal-C2-Cer (**5**), and C2-Cer (**1**) on allogeneic MLR. Murine spleen cells were cultured for 4 days, and the proliferation of spleen cells was measured by the 8-h [^{3}H]TdR pulse method. Experiments were performed in triplicate, and the mean with SD is shown.

0.063−0.200 mm). TLC analyses were done on silica gel plates (Merck, art. 5554). All melting points were measured on a Yanagimoto micromelting point apparatus and are uncorrected. Mass spectra were measured on a JEOL JMS SX/SX-102 mass spectrometer. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. Elemental analyses were recorded with a Perkin-Elmer 240C elemental analyzer. ^{1}H NMR spectra were obtained using a JEOL JNM-GX-500 FT NMR spectrometer; chemical shifts are expressed in *δ* units from tetramethylsilane (TMS) as an internal standard, and coupling constants (*J*) are reported in hertz (Hz).

(2*S*,3*R*,4*E*)-2-Amino-1-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-*N*-acetyl-4-octadecene-1,3-diol (3). To a stirred solution of C2-Cer (78 mg, 0.23 mmol) in THF (5 mL) were added stannous chloride (110 mg, 0.58 mmol), silver perchlorate (110 mg, 0.53 mmol) and molecular sieves 4A powder (250 mg), and the mixture was stirred at room temperature for 30 min. The mixture was cooled to −10 °C, and a solution of benzylgalactosyl fluoride[11] (**2**; 187 mg, 0.35 mmol) in THF (1 mL) was added dropwise. Then the resulting mixture was allowed to warm to room temperature and stirred for an additional 2 h. The mixture was diluted with acetone and filtered through Celite. Then the filtrate was concentrated. The residue was dissolved in EtOAc, washed with brine, dried over $MgSO_4$, and then concentrated in vacuo. The residue was purified by chromatography on a silica gel column (10 g) using hexanes−EtOAc (6:1) as the eluent to give α-galactoside **3**: 45 mg (23%); mp 88.0−89.0 °C; $[\alpha]^{23}_D$ +32.4° (*c* 0.1, $CHCl_3$); FDMS *m*/*z* 864 M^+; ^{1}H NMR (500 MHz, $CDCl_3$) *δ* 7.26−7.35 (20H, m), 6.45 (1H, d, *J* = 7.9 Hz), 5.62−5.69 (1H, m), 5.42 (1H, dd, *J* = 5.5, 15.3 Hz), 4.91 (1H, d, *J* = 11.6 Hz), 4.85 (1H, d, *J* = 11.6 Hz), 4.74−4.80 (3H, m), 4.70 (1H, d, *J* = 11.6 Hz), 4.56 (1H, d, *J* = 11.6 Hz), 4.47 (1H, d, *J* = 11.6 Hz), 4.38 (1H, d, *J* = 11.6 Hz), 4.10−4.18 (1H, m), 3.95−4.08 (3H, m), 3.80−3.92 (3H, m), 3.73 (1H, d, *J* = 9.8 Hz), 3.70 (1H, dd, *J* = 3.7, 10.4 Hz), 3.45−3.57 (2H, m), 1.99 (2H, dt, *J* = 3.4, 7.3 Hz), 1.93 (3H, s), 1.20−1.40 (22H, m), 0.88 (3H, t, *J* = 6.7 Hz). Anal. ($C_{54}H_{73}NO_8$) C, H, N.

(2*S*,3*R*,4*E*)-2-Amino-1-*O*-(α-D-galactopyranosyl)-*N*-acetyl-4-octadecene-1,3-diol (4). To liquid NH_3 (ca. 8 mL) under N_2 at −78 °C was added Na (20 mg), and the mixture was stirred for 2 min. To the blue solution was added **3** (40 mg, 46.3 *μ*mol) in THF (1 mL), and the mixture was stirred for 40 min at −78 °C. The reaction was quenched by addition of MeOH (4 mL). Ammonia was removed with a stream of N_2, and then the solution was diluted with MeOH to ca. 8 mL. The reaction mixture was neutralized with Dowex 50-X8 (200 mg), filtered, rinsed with NH_3 in MeOH (30 mL), and concentrated in vacuo. The residue was purified by chromatography on a silica gel column (10 g) using $CHCl_3$−MeOH (5:1) as the eluent to give α-galactoside **4**: 17 mg (73%); mp 144.0−146.0 °C; $[\alpha]^{23}_D$ +47.6° (*c* 0.1, pyridine); FDMS *m*/*z* 504 M^+; ^{1}H NMR (500 MHz, C_5D_5N) *δ* 8.58 (1H, d, *J* = 8.6 Hz), 5.95−6.00 (2H, m), 5.43 (1H, d, *J* = 3.7 Hz), 4.78−4.89 (2H, m), 4.64 (1H, dd, *J* = 3.7, 9.8 Hz), 4.56 (1H, d, *J* = 3.1 Hz), 4.51 (1H, t, *J* = 6.1 Hz), 4.40−4.48 (4H, m), 4.31 (1H, dd, *J* = 5.5, 10.4 Hz), 2.10 (3H, s), 2.06 (2H, dt, *J* = 3.4, 7.3 Hz), 1.20−1.40 (22H, m), 0.87 (3H, t, *J* = 6.7 Hz). Anal. ($C_{26}H_{49}NO_8$) C, H, N.

Biological Methods. Animals: Female C57BL/6 or BALB/c mice, 5−10 weeks old, purchased from Nippon SLC Co., Ltd., were used in this study. Mice were maintained under our standard laboratory conditions.

Preparation of Spleen Cells: Mice were sacrificed, and the spleens were resected. The spleens were dissociated in 10% fetal calf serum (FCS; Gibco) RPMI 1640 (Gibco) medium, and RBCs were lysed with Tris NH_4Cl. The cells were washed three times using phosphate-buffered saline (Nissui Pharmaceutical Co., Ltd.), and viable cells were counted and resuspended in 10% FCS RPMI 1640 medium.

Allogeneic MLR Assay: Spleen cells obtained from BALB/c mice were used as responder cells, and MMC (50 *μ*g/mL·30 min)-treated spleen cells from C57BL/6 mice were used as stimulator cells in allogeneic MLR assay. Same volume (1×10^5 cells/50 *μ*L/mL) of responder cells and stimulator cells suspended in 10% FCS RPMI 1640 medium were plated in 96-well round-bottomed plates (Nunclon). At the same time, 10 *μ*L/well control diluent (10% DMSO in PBS) or C2-Cer and its derivatives (0.8, 4, and 20 *μ*g/mL) were added in the 96-

well plate. The plate was cultured at 37 °C in 95% air, 5% CO_2. Four days later, 0.5 μCi/well tritium thymidine ([^{3}H]TdR; DuPont/NEN Research Products) was added into each well and the plate was incubated for an additional 8 h. [^{3}H]TdR uptake into cells was measured using a liquid scintillation counter.

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