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Enhancing Effects of α-, β-Monoglycosylceramides on Natural Killer Cell Activity

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Abstract—We examined in vitro and in vivo natural killer (NK) cell activity enhancing effects of α-, β-galactosylceramide (GalCer) and α-, β-glucosylceramide (GluCer) which have the same ceramide moiety, and of other α-, β-GalCer having a different ceramide portion, and found that α-types show stronger enhancing effects than β-types and the α-GalCer possesses the most potent activity among GalCers and GluCers having the same ceramide moiety. When the comparison of tumor growth inhibitory effects of α-, β-GalCer on mice subcutaneously inoculated with B16 cells was performed, the α-GalCer showed stronger suppressive activity than its β-type, paralleling their enhancing effects on NK cell activity. These results suggest that the manner of combination between sugar and ceramide plays an important role in antitumor activity as well as enhancing effect on NK cell activity of GalCers. Copyright © 1996 Elsevier Science Ltd

Introduction

We previously examined the allogenic mixed leukocyte reaction (MLR), stimulatory and antitumor activities of four kinds of α-, β-GalCers and α-, β-GluCers having the same ceramide structure synthesized in our laboratory and found that the α-GalCer and α-GluCer showed stronger biological activities than their β-types, and the potencies of α-GalCer were stronger than those of α-GluCer.[1,2] These results suggest that the manner of combination between sugar and ceramide and the type of sugar combined to ceramide play an important role in the manifestation of biological activities of monoglycosylceramides (MonoCers). **KRN7000** which is an α-GalCer was selected as a candidate for clinical application by the study of the structure–activity relationship of α-GalCers against mice subcutaneously inoculated with murine malanoma B16 cells.[3] The study of the mechanisms of action of **KRN7000** showed that **KRN7000** markedly activates natural killer (NK) cells which are antitumor effector cells in vitro and in vivo, and the antitumor activity of **KRN7000** is caused by the activation of NK cells.[4]

The results raised the question whether the manner of combination between sugar and ceramide and the type of sugar combined to ceramide also play an important role in the enhancing effects of MonoCers on NK cell activity. To address this question, we performed the following experiments. In this paper, we describe the enhancing effects of α-, β-GalCers and α-, β-GluCer on in vitro and in vivo NK cell activity and the relationship between their enhancing effects and spleen cell-proliferation stimulatory activities.

Key words: **KRN7000**, natural killer cells, galactosylceramide, glycosylceramide, immunomodulator.

Results and Discussion

We previously demonstrated that α-GalCer and α-GluCer show stronger spleen cell-proliferation stimulatory effects on allogenic MLR than their β-types which have the same ceramide moiety.[2] To examine the relationship between their allogenic MLR stimulatory activities, and their enhancing effects on NK cells, we first examined in vitro NK cell activity enhancing effects of six kinds of MonoCers synthesized in our laboratory.[5] Four of the MonoCers kinds were α- and β-GalCer (**AGL-517** and **AGL-564**, respectively) and α- and β-GluCer (**AGL-563** and **AGL-562**, respectively) which have the same ceramide portion. The others, α- and β-GalCer (**KRN7000** and **AGL-583**, respectively) have a different ceramide moiety from **AGL-517**. Structures are shown in Figure 1.

As Table 1 shows, **AGL-517** (α-GalCer) concentration-dependently enhanced the in vitro NK cell activity and its potency was stronger than that of its β-type (**AGL-564**). In the case of GluCer, a similar relationship was observed, and **AGL-563** (α-GluCer) showed stronger enhancing activity than **AGL-562** (its β-type). In addition, the α-GalCer showed stronger enhancing effect than the α-GluCer when they had the same ceramide portion. It should be noted that the above-mentioned relationship among the four kinds of MonoCers resembles their allogenic MLR stimulatory activities.[2] Furthermore, we compared the enhancing effects of **KRN7000** and **AGL-583** in order to confirm

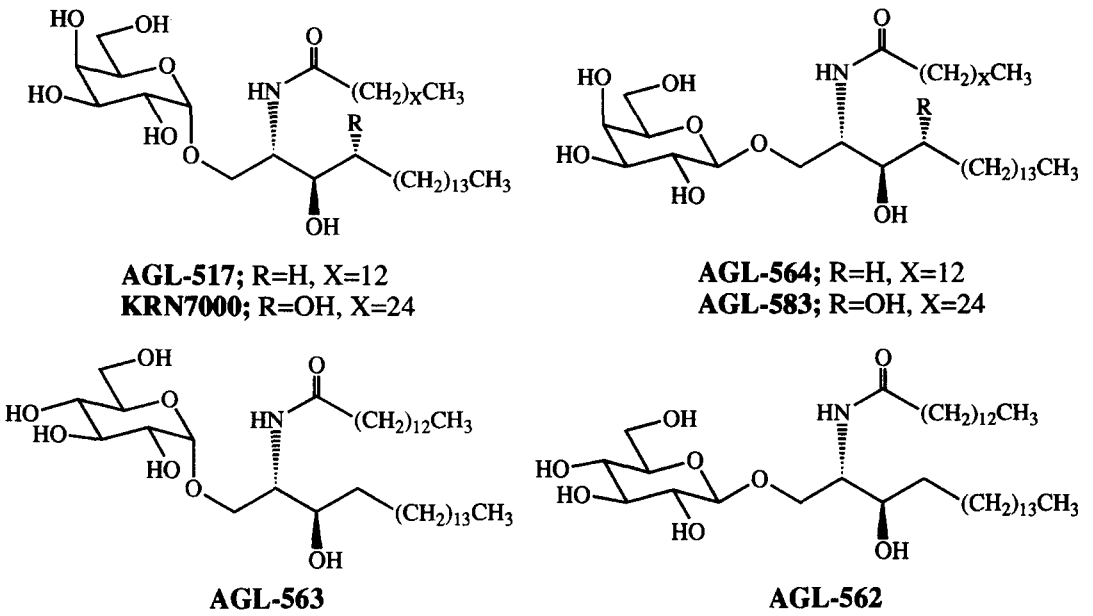

Figure 1. Structures of **KRN7000**, **AGL-517**, **AGL-583**, **AGL-564**, **AGL-563** and **AGL-562**.

that α-GalCers show stronger enhancement of NK cell activity than β-GalCers having the same ceramides, and found that **KRN7000** markedly enhances the in vitro NK cell activity and its potency is stronger than that of **AGL-583**.

We previously demonstrated that the allogenic MLR stimulatory activities of α-GalCers parallel their proliferation stimulatory effects on spleen cells.[3] Using six kinds of MonoCers, we examined the relationship between their enhancing effects on in vitro NK cell activity and their spleen cell-proliferation stimulatory activities. As shown in Table 2, α-GalCers and α-GluCer stimulated the proliferation more markedly than their β-types and the potencies of α-GalCers were stronger than those of α-GluCer, paralleling their enhancing effects on in vitro NK cell activity. These results demonstrated that not only the manner of combination between sugar and ceramide, but also the type of sugar combined to ceramide plays an important role in the manifestation of spleen cell-proliferation stimulatory activities, allogenic MLR stimulatory activities, and in vitro enhancing effects on NK cell activity of MonoCers.

Since it has been suggested that the antitumor activies of **KRN7000** originate in its enhancing effect on in vivo NK cell activity,[4] we then evaluated the enhancing activities of the six kinds of MonoCers against in vivo NK cell activity in order to examine the relationship between their enhancing effects and antitumor activities. As shown in Figure 2, **KRN7000**, **AGL-517** and **AGL-563** showed stronger enhancing activities than **AGL-583**, **AGL-564** and **AGL-562** which are their respective β-types, and the potency of **KRN7000** was much stronger than that of Poly I:C which is a positive control.[6] In addition, the enhancing activity of **AGL-517** (α-GalCer) was stronger than that of **AGL-563** (α-GluCer). These results resemble their enhancing effects on in vitro NK activity (Table 1) and their proliferaion stimulatory activities against spleen cells (Table 2).

Table 1. Enhancing effects of the six kinds of MonoCers on in vitro NK cell activity

Sample	% of Specific lysis (mean ± SD)		
	1 (ng/mL)	10 (ng/mL)	100 (ng/mL)
Vehicle	4.4 ± 2.5	4.8 ± 2.6	3.5 ± 1.8
AGL-517	4.4 ± 1.2	18.7 ± 2.7	30.1 ± 1.4
AGL-564	3.5 ± 0.3	3.9 ± 1.4	4.2 ± 0.7
AGL-563	3.6 ± 0.7	8.0 ± 2.1	18.7 ± 5.1
AGL-562	3.7 ± 2.3	3.8 ± 0.4	4.0 ± 0.9
KRN7000	12.2 ± 2.0	13.8 ± 0.6	30.6 ± 3.1
AGL-583	3.7 ± 1.2	3.4 ± 0.7	3.3 ± 1.0

Murine spleen (effector) cells (2.5×10^5 cells/100 μL/well) were plated in 96-well plates in triplicate and cultured with 1–100 ng/mL of MonoCers for 4 days at 37°C in 95% air, 5% CO_2. ^{51}Cr-labeled YAC-1 (target) cells (1×10^4 cells/well) were added to each well, and the plate incubated for a further 4 h. ^{51}Cr release from lysed target cells was measured using a γ-counter.

Table 2. Spleen cell-proliferation stimulatory effects of the six kinds of MonoCers

Sample	^{3}H-TdR incorporation (cpm, mean ± SD)		
	1 (ng/mL)	10 (ng/mL)	100 (ng/mL)
Vehicle	3016 ± 455	3115 ± 679	2325 ± 116
AGL-517	6531 ± 184	15333 ± 2336	24117 ± 1533
AGL-564	2737 ± 212	2577 ± 341	2856 ± 109
AGL-563	3422 ± 333	4662 ± 317	10528 ± 1108
AGL-562	2833 ± 323	2526 ± 258	2387 ± 548
Vehicle	2627 ± 330	2664 ± 344	2517 ± 491
KRN7000	8959 ± 234	9998 ± 551	21075 ± 1481
AGL-583	2483 ± 273	2444 ± 286	2456 ± 587

Murine spleen cells (2.5×10^5 cells/100 μL/well) were plated in 96-well plates in triplicate and cultured with 1–100 ng/mL of MonoCers at 37°C in 95% air, 5% CO_2. Two days after ^{3}H-TdR were added to each well, and the plate was incubated for a further 8 h. ^{3}H-TdR uptake into cells was counted using a liquid scintillation counter.

It has been reported that activated NK cells play an important role in the prevention of cancer growth and metastasis.[7] Furthermore, the enhancing effects of MonoCers (**AGL-517**, **AGL-564**, **AGL-563** and **AGL-562**) on in vivo NK cell activity seem to resemble their antitumor activities against mice subcutaneously inoculated with B16 or Meth A cells.[1] The above-mentioned results seem to support our hypothesis that the potent antitumor activities of α-GalCers such as **KRN7000** originate in their potent enhancing effects on in vivo NK cell activity. To confirm this hypothesis, we compared tumor growth inhibitory effects of **KRN7000**, which showed the most potent activation of NK cells, and **AGL-583** on mice subcutaneously inoculated with B16 cells. As Figure 3 shows, **KRN7000** markedly suppressed the tumor growth, and its activity was stronger than that of **AGL-583**, paralleling their enhancing effects on in vivo NK cell activity. In addition, when **KRN7000** was administered to mice in combination with Mitomycin C (MMC), which is a chemotherapeutic agent and used as a positive control,[8] the stronger inhibitory effect which seems to be an additive effect was observed in comparison with those of single treatments. In contrast, the combined treatment of **AGL-583** and MMC did not show any additive or synergetic effects. The result is considered to support our hypothesis.

Conclusion

In this paper, we have described that not only the manner of combination between sugar and ceramide but also the type of sugar combined to ceramide play an important role in enhancing activities of MonoCers against in vitro and in vivo NK cell activities, and that α-GalCer shows the strongest activities among GalCers and GluCers which have the same ceramide portion.

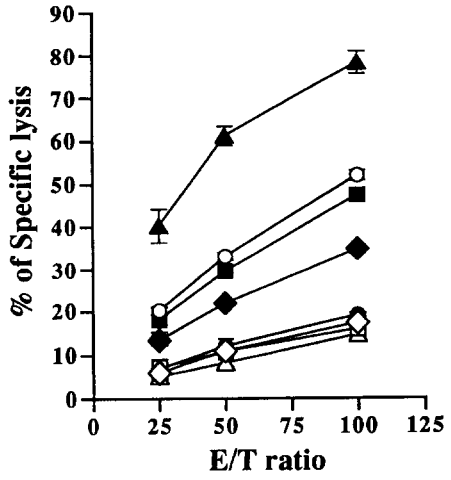

Figure 2. Enhancing effects of the six kinds of MonoCers on in vivo NK cell activity. Spleen cells from mice intravenously treated with the six kinds of MonoCers (100 μg/kg) or Poly I:C (2 mg/kg) 2 days before use as effector cells. These spleen cells and ^{51}Cr-labeled YAC-1 (target) cells were plated in triplicate to produce E:T ratios of 100:1, 50:1 and 25:1, and their lytic activities were measured by the 4 h-^{51}Cr-release method. Each point represents the mean ± SD of three wells. (●) Vehicle, (▲) **KRN7000**, (△) **AGL-583**, (■) **AGL-517**, (□) **AGL-564**, (◆) **AGL-563**, (◇) **AGL-562**, (○) Poly I:C.

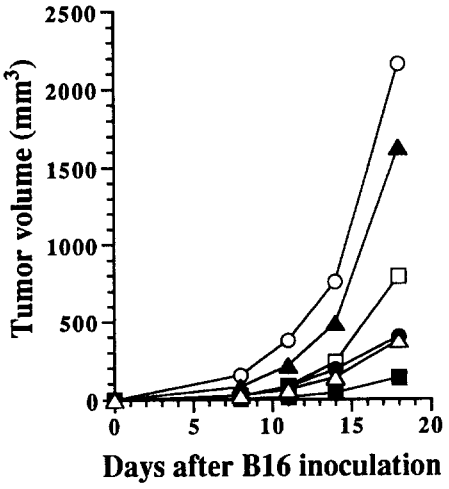

Figure 3. Tumor growth inhibitory effects of **KRN7000** and **AGL-583** in combination with or without MMC on mice subcutaneously inoculated with B16 cells. B16 cells (1×10^6 cells/mouse) were subcutaneously inoculated into mice on day 0. **KRN7000** and **AGL-583** (100 μg/kg) were intravenously administered on days 1, 5 and 9. MMC (5 mg/kg) was intravenously given on day 1. Each tumor volume per mouse was measured using callipers on days 8, 11, 14 and 18. Each point represents the mean of five mice. (○) Vehicle, (□) **KRN7000**, (▲) **AGL-583**, (●) MMC, (■) MMC+**KRN7000**, (△) MMC+**AGL-583**.

Furthermore, it was strongly suggested that the enhancing effects of the MonoCers resemble their allogenic MLR stimulatory activities, their spleen cell-proliferation stimulatory effects, and their antitumor activities. Although various types of β-GalCers and β-GluCers have been isolated from organ tissues[9,10] and marine organisms,[11-15] the marked biological activities mentioned above have not yet been reported. Our results seem to show the reason why there are no reports concerning the immunostimulatory and antitumor activities of β-GalCers and β-GluCers.

Experimental

Syntheses and physical properties of MonoCers

The synthetic procedures for the six kinds of MonoCers used in this paper and their physical properties have been previously reported.[5]

Animals

Female mice of BDF_1 strain, 5–8 weeks old, purchased from Nippon SLC Co., Ltd were used in these studies. 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), and RBC were lysed with Tris–NH_4Cl. The cells were washed three times using phosphate buffer saline (Nissui Pharmaceutical Co., Ltd), and viable cells were counted and resuspended in 10% FCS RPMI 1640.

Spleen cell-proliferation assay

Spleen cells (2.5×10^5 cells/100 μL/well) suspensed in 10% FCS RPMI 1640 medium and 1, 10 and 100 ng/mL of MonoCers (10 μL/well) were plated in 96-well round-bottomed plates (Nunclon) and cultured at 37 °C in 95% air, 5% CO_2. Two days later, 0.5 μCi/well of tritium–thymidine (^{3}H-TdR, Du Pont/NEN Research Products) was added into each well and the plate was incubated for a further 8 h. ^{3}H-TdR uptake into cells was measured using a liquid scintillation counter.

In vitro NK activity assay

Spleen cells (2.5×10^5 cells/100 μL/well) suspensed in 10% FCS RPMI 1640 medium and 1, 10 and 100 ng/mL of MonoCers (10 μL/well) were plated in 96-well round-bottomed plates and cultured at 37 °C in 95% air, 5% CO_2. Four days later, these spleen cells were used as effector cells. YAC-1 lymphoma (target) cells were labeled with 100 μCi sodium [^{51}Cr] chromate (Du Pont/NEN Research Products)/5×10^6 cells for 1 h, washed three times, and resuspended at 1×10^6 cells/mL of medium. YAC-1 cell suspension (10 μL) was added to each well of 96-well round-bottomed plate. Plates were incubated for 4 h at 37 °C in 95% air, 5% CO_2, and ^{51}Cr release from lysed target cells was measured using a γ-counter. The percentage of specific chromium released at each well was computed using the following formula: % of specific lysis = [(Test CPM − Spontaneous CPM)/(Total CPM − Spontaneous CPM)] × 100. Where; Test CPM = counts in experimental cultures of target cells and effector cells; Spontaneous CPM = counts in cultures containing only target cells and 100 μL of medium; Total CPM = counts obtained by adding 100 μL of 1 N HCl to target cells to lyse all cells.[16]

In vivo NK activity assay

In vivo NK cell assay was performed by the following method. Briefly, spleen cells from mice (3 mice per group) intravenously treated with 100 μg/kg of MonoCers or 2 mg/kg of Poly I:C (Sigma) 2 days before were used as effector cells. ^{51}Cr-labeled target (YAC-1) cells were prepared with the above-mentioned method. Spleen (effector) cells were added to 96-well round-bottomed plates to produce effector to target (E:T) ratios of 100:1, 50:1 and 25:1, and 10 μL of ^{51}Cr-labeled target cell suspension (1×10^4 cells/well) was added to each well. Plates were incubated for 4 h at 37 °C in 95% air, 5% CO_2, and ^{51}Cr release from lysed target cells was measured using a γ-counter.[6] The percentage of specific chromium released at each well at each E:T ratio was computed using the above-mentioned formula.

Tumor growth inhibitory effects of KRN7000 and AGL-583 in combination with or without MMC

Five female BDF_1 mice per group were used. B16 cells (1×10^6 cells/mouse) were subcutaneously inoculated into mice on day 0. **KRN7000** and **AGL-583** (100 μg/kg) were intravenously administered on days 1, 5 and 9. MMC (5 mg/kg) was intravenously given on day 1. Each tumor volume (length × width × height/2, mm^3) per mouse was measured using callipers on days 8, 11, 14 and 18.

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