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Natural Killer T Cell Ligand α-Galactosylceramide Inhibited Lymph Node Metastasis of Highly Metastatic Melanoma Cells

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The role of natural killer T (NKT) cells in the prevention of multiple tumor metastasis was examined. The i.v. inoculation of a highly metastatic subline of B16-BL6 (B16-BL6-HM) melanoma cells resulted in the formation of metastatic nodules in lymph nodes in addition to lung, intrapleural cavity, and ovary. However, treatment of the mice with the NKT cell ligand α-galactosylceramide (α-GalCer) three times from 1 day after B16-BL6-HM melanoma inoculation caused a significant inhibition of multiple metastasis. Lymph node metastasis of B16-BL6-HM was almost completely blocked by α-GalCer treatment. This antimetastatic effect of α-GalCer was abolished in NKT cell-deficient mice. These results suggest that α-GalCer-activated NKT cells played a critical role in the prevention of lymph node metastasis of melanoma cells.

Key words: NKT — α-galactosylceramide — Metastasis — Melanoma — Immunotherapy

Immunesurveillance mechanisms, involving both innate and acquired immunity, play a critical role in the prevention of tumor growth and metastasis *in vivo*.[1, 2] It has been demonstrated that $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ helper T cells (Th), which are involved in acquired immunity, are essential for the induction of complete tumor rejection and for the acquisition of immunological memory of the rejected tumor antigen.[3] However, it has also been demonstrated that natural killer T (NKT) cells, which are one of the effector cells in innate immunity, as well as natural killer (NK) cells, show a potent antitumor activity *in vivo*.[4, 5] NKT cells, distinct from other lymphoid cells including T cells, B cells, and NK cells, are characterized by coexpression of the NK receptor and a single, invariant T cell receptor (TCR) encoded by Vα14 and Jα281 gene segments.[6] In contrast to mainstream $CD4^+$ T cells, $CD4^+NK1.1^+$ NKT cells can recognize glycolipid antigen on CD1d molecules of dendritic cells (DC) and produce cytokines which accelerate the induction of acquired immune responses mediated by CTL and Th.[4, 7]

Recently, a ligand for $V\alpha14^+$ NKT cells was defined as α-galactosylceramide (α-GalCer), which can stimulate both cytotoxicity and cytokine production of NKT cells in the presence of DC.[8, 9] Using this novel NKT-specific immunopotentiator, it became possible to evaluate the role of NKT cells in tumor immunity. Indeed, it has been clearly demonstrated that α-GalCer treatment caused marked inhibition of tumor metastasis to liver[5] and lung.[4] Moreover, Kawano *et al.*[4] demonstrated that the antimetastatic activity of α-GalCer is totally dependent on NKT cells based on the finding that α-GalCer showed no significant antitumor activity in NKT cell-deficient mice. These data strongly suggested that NKT cells play an important role in the inhibition of hematogenous tumor metastasis into liver and lung. However, it still remains unclear whether NKT cells can also prevent distant lymph node metastasis, which is clinically a major determinant of poor prognosis.

To evaluate the role of NKT cells in lymph node metastasis, we examined the therapeutic effect of the NKT cell ligand α-GalCer on lymph node metastasis of a highly metastatic subline of B16-BL6 (B16-BL6-HM) melanoma cells. A multiple metastasis model was established by i.v. injection of B16-BL6-HM cells (10^5) into C57BL/6 mice. The therapeutic effect of α-GalCer (2 μg/mouse) was examined by i.v. injection into the mice 3 times from the day after tumor inoculation. Two weeks after B16-BL6-HM inoculation, the mice treated with or without α-GalCer were killed and the numbers of metastatic nodules in various organs were counted to determine the therapeutic effect of α-GalCer on tumor metastasis.

As shown in Fig. 1A, α-GalCer injection into the tumor-inoculated mice for 3 days caused a marked elevation of serum interferon (IFN)-γ levels. Although interleu-

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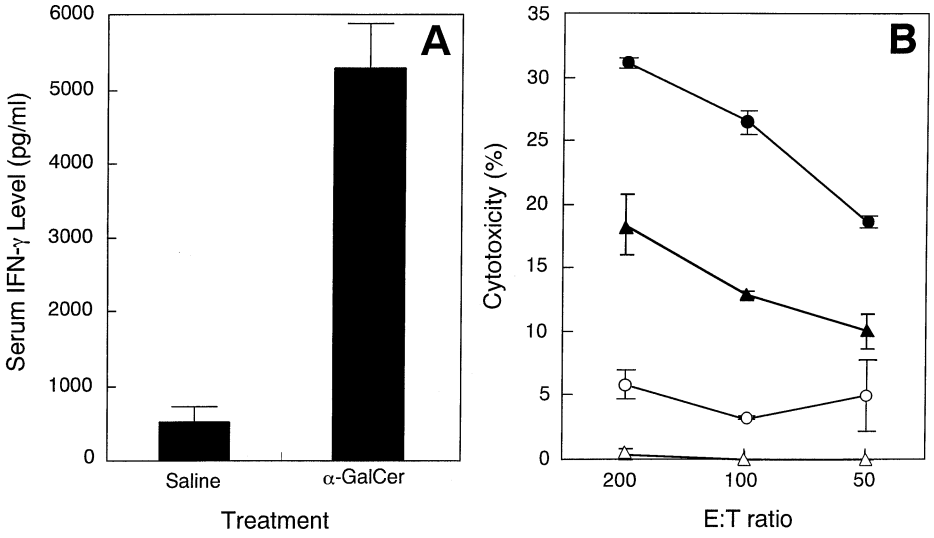

Fig. 1. Immunopotentiating effect of α-galactosylceramide on natural killing activity and IFN-γ production. C57BL/6 mice were i.v. inoculated with 10^5 B16-BL6-HM. A day after tumor inoculation, the mice were i.v. treated with α-GalCer (2 μg/mouse) 3 times. Control mice were treated with saline. A, The mice were killed one day after final treatment with α-GalCer or saline and their serum IFN-γ activity was measured by ELISA. B, Spleen cells were prepared from the mice treated with α-GalCer (●, ▲) or saline (○, △) and their natural killing activity against YAC-1 cells (●, ○) or cytotoxicity against B16-BL6-HM (▲, △) was determined by 4-h ^{51}Cr-release assay as described previously.[12] The mean±SE of three samples is shown.

kin (IL)-4 and IL-12 were not detected 24 h after α-GalCer-treatment, a significant elevation of serum levels of IL-4 (3155±220 pg/ml) and IL-12 (95.2±23 pg/ml) was detected at 4 h after the α-GalCer-treatment in accordance with previous results.[7] Moreover, as shown in Fig. 1B, α-GalCer-treatment of tumor-inoculated mice caused the activation of natural killing activity of spleen cells against YAC-1 and B16-BL6-HM cells. Although a slight augmentation of natural killing activity towards YAC-1 (7.1%) and B16-BL6-HM (2.5%) was also demonstrated in lymph node cells, their activity was much lower than that of spleen cells. Therefore, the activation of NKT cells by α-GalCer might be preferentially induced in spleen compared with lymph nodes.

In parallel with these immunopotentiating effects, α-GalCer-treatment caused a great reduction of B16-BL6-HM nodules in lung and completely abolished axillary lymph node metastasis, though saline-treated mice formed multiple metastases in lung, lymph nodes (axillary, mesenteric), intrapleural cavity and ovary (Fig. 2, A, B and C). As shown in Fig. 2, B and C, a partial prevention of lung metastasis was observed. However, lymph node (axillary) metastasis was almost completely inhibited by α-GalCer administration (Fig. 2C). The effect of α-GalCer administration on lymph node metastasis is summarized in Table I. The existence of metastatic nodules in the right axilla, left axilla and mesenterium was examined 2 weeks after B16-BL6-HM inoculation. Over 90% of mice exhibited B16-BL6-HM metastasis into lymph nodes. However, lymph node metastasis of B16-BL6-HM was strongly blocked by α-GalCer administration and all or 9 out of 10 mice were completely free from metastatic tumor nodules in axillary or mesenteric lymph nodes (Table I). The metastasis of B16-BL6-HM into the intrapleural cavity and ovary was also blocked by α-GalCer-treatment (data not shown).

To demonstrate that α-GalCer-induced antimetastatic activity was mediated by NKT cells, the therapeutic effect of α-GalCer was also examined using NKT-deficient Jα281$^{-/-}$ mice.[4, 10] The results are also shown in Table I. The therapeutic effect of α-GalCer on lymph node metastasis was completely abolished in NKT-deficient mice and 100% of NKT-deficient mice inoculated i.v. with B16-BL6 melanoma developed lymph node metastasis. The antimetastatic activity of α-GalCer on lung, kidney and intrapleural cavity was also abolished in NKT-deficient mice (data not shown). From these data, we concluded that α-GalCer-activated NKT cells played a pivotal role in the prevention of lymph node metastasis of the tumor, in addition to hematogenous metastasis.

Recently, Kawano *et al.*[4] demonstrated that α-GalCer-induced antitumor activity *in vivo* is totally dependent on both NKT cells and CD1d$^+$ DC. We further demonstrated that α-GalCer showed an immunopotentiating effect through the activation of IL-12 production of DC and IL-12 receptor induction of NKT cells.[7] These previous results and our present finding that α-GalCer showed a

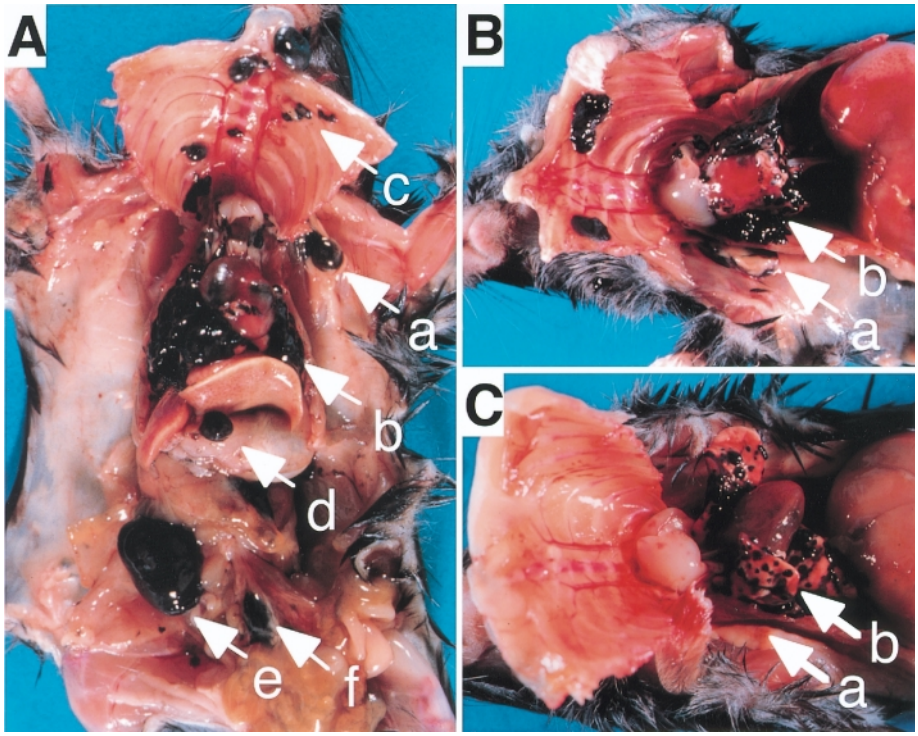

Fig. 2. Therapeutic effect of α-GalCer on multiple metastasis of B16-BL6-HM melanoma cells in C57BL/6 mice. Highly metastatic B16-BL6-HM melanoma cells were selected from metastatic nodules of B16-BL6 by repeated *in vivo* passage. A, B16-BL6-HM showed multiple metastasis into axillary lymph node (a), lung (b), intrapleural cavity (c), pyloric lymph node (d), ovary (e), mesenteric lymph node (f) 2 weeks after i.v. tumor inoculation. B, Control mice bearing metastatic B16-BL6-HM tumor nodules in axillary lymph node (a) and lung (b). C, The inhibition of B16-BL6-HM tumor metastasis into axillary lymph node (a) and lung (b) by α-GalCer.

Table I. α-GalCer Inhibited Lymph Node Metastasis of B16-BL6-HM Melanoma Cells in NKT-cell Dependent Manner

Mice[a)]	Treatment[b)]	Right axilla[c)]	Left axilla	Mesenterium
C57BL/6	Saline	9/10	10/10	9/10
	α-GalCer	0/10	0/10	1/10
Vα14 NKT-deficient	Saline	5/5	5/5	5/5
	α-GalCer	5/5	5/5	5/5

a) C57BL/6 wild-type mice or Vα14 NKT-deficient mice were used for the experiment.
b) The mice were i.v. inoculated with 10^5 B16-BL6-HM melanoma cells. Then, the mice were treated with saline or α-GalCer for 3 days from the day after tumor inoculation.
c) The existence of lymph node metastasis in right axilla, left axilla and mesenterium was examined 2 weeks after B16-BL6-HM tumor inoculation. Ten wild-type mice and 5 NKT-deficient mice were used for the experiment. The number of mice bearing metastatic nodules/number of mice used for the experiment is shown.

potent inhibitory effect on both hematogenous and lymph node metastasis of tumor suggest that DC and NKT cells, both of which are involved in innate immunity, play an important role in the prevention of lymph node metastasis. Recently, NK cells in RAG2$^{-/-}$ mice were also demonstrated to be effective in blocking tumor metastasis.[11)] Therefore, both NK and NKT cells might be involved in the inhibition of tumor metastasis *in vivo* during the early tumor-bearing stage. However, innate effector cells such as NKT cells appeared to be ineffective against an intra-

dermally injected-tumor mass, because a B16-BL6-HM tumor mass was not rejected after α-GalCer-treatment though it was completely eradicated by IL-12-injection (data not shown). Therefore, a regimen that activates both NKT cells, CTL, and Th cells should provide a superior therapeutic effect in systemic therapy of a solid tumor mass in addition to tumor metastasis. For this purpose, we are currently investigating the combined therapeutic effect of α-GalCer and IL-12 against multiple tumor metastasis and solid tumor mass.

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