Antitumor Cytotoxicity Mediated by Ligand-activated Human Vα24 NKT Cells

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Abstract

Human Vα24 NKT cells bearing an invariant Vα24JOQ antigen receptor, the counterpart of the murine Vα14 NKT cells, are activated by the specific ligand, α-galactosylceramide (α-GalCer) in a CD1d-dependent manner. Here, we demonstrate that the α-GalCer-activated Vα24 NKT cells exert a potent perforin-dependent cytotoxic activity against a wide variety of human tumor cell lines. In addition, we demonstrate that Vα24 NKT cells and dendritic cells (DCs) from melanoma patients are functionally normal, even in the tumor-bearing status. The potential use of α-GalCer-activated Vα24 NKT cells and/or DCs from patients for cancer immunotherapy is discussed.

Introduction

The murine Vα14 NKT cells recognize a glycolipid, α-GalCer, in a CD1d-dependent fashion (1, 2) and display a potent NK-like cytotoxic activity against various tumor cell lines (3). The activated Vα14 NKT cells are also shown to inhibit tumor metastasis in certain experimental animal models (3). Human NKT cells bearing the invariant Vα24JOQ receptor are considered to be the counterpart of murine Vα14 NKT cells and may recognize similar antigens to those of the murine Vα14 NKT cells, because of the striking homology between human Vα24 and mouse Vα14 receptors, particularly in their CDR3 region (4). In fact, human Vα24 NKT cells have been found to be activated by α-GalCer in a CD1d-dependent fashion (5–7); thus, it is conceivable that human Vα24 NKT cells could also develop antitumor activity upon α-GalCer stimulation. Here, we show that Vα24 NKT cells from PBLs of patients with malignant melanoma can be activated by α-GalCer and display a potent perforin-dependent cytotoxic activity. Also, the ability of DCs to present α-GalCer is found to be preserved in melanoma patients. These findings raise the possibility that this “α-GalCer/CD1-NKT cell system” could become a new effective tool for cancer immunotherapy.

Materials and Methods

In Vitro Activation and Expansion of Vα24 NKT Cells. Vα24 NKT cells were purified from umbilical cord blood or PBLs of healthy volunteers and patients with malignant melanomas after obtaining the informed consent (7). FITC-conjugated anti-Vα24 monoclonal antibody (C15; Coulter-Immunotech, Miami, FL) and anti-FITC magnetic beads (MACS; Miltenyi Biotech, Gladbach, Germany) were used for separation. For the preparation of APCs, CD3− cells (purity >99%) were purified by negative selection by MACS. Enriched Vα24 NKT cells (1 × 106) and CD3+ APCs (1 × 106) were cocultured in the presence of α-GalCer (10 ng/ml; KRN7000, Kirin Brewery Co. Tokyo, Japan) and recombinant human IL-2 (100 units/ml; Boehringer Mannheim, Mannheim, Germany).

Flow Cytometry Analysis. The percentages of Vα24 NKT cells and the expression levels of MHC class I molecules were evaluated by flow cytometry analysis (7) with specific antibodies as follows; anti-Vα24 (C15); anti-Vβ11 (C21; Immunotech); anti-CD3 (UCHT-1; Pharmingen, La Jolla, CA); and pan-reactive anti-class I antibody (G46–2.6; Pharmingen).

Measurement of Cytotoxic Activity of Activated Vα24 NKT Cells against Human Tumor Cell Lines. Cytotoxicity of α-GalCer-activated Vα24 NKT cells was measured by a standard 4-h 3Cr-release assay (3) on various human tumor cell lines; Daudi B lymphoma, Molt-4 T lymphoma, and K562 myelogenous leukemia; SK-Mel-28 and BM-1 malignant melanoma; PC10 squamous cell lung carcinoma; PC6 small cell lung carcinoma; PC13 large cell lung carcinoma, and ABC-1 lung adenocarcinoma; Alexander hep- atoma; RCM-1 colon adenocarcinoma; Panc-1 pancreas carcinoma; HeLa uterine cervical carcinoma; SHIN-3 ovarian adenocarcinoma; and TN-1 neon- roblastoma. Con A blasts of human PBLs were also used as targets. Treatment of Vα24 NKT cells with concanamycin A (Wako Pure Chemical, Tokyo, Japan) was performed as described (3).

Evaluation of Antitumor Effects of Vα24 NKT Cells in Vivo. A human esophageal cancer T.Tn cell line (1 × 107) was inoculated s.c. into the back of BALB/c nude mice (SLC, Shizuoka, Japan) with or without activated Vα24 NKT cells (1 × 107) according to the protocol described by Winn (Ref. 8; Winn’s assay). IL-2 (5000 units) was administrated i.p. once a day from days 0 to 4 as described (9–11).

Evaluation of the α-GalCer Presentation of DCs of Melanoma Patients. Human DCs were prepared from PBLs of melanoma patients. Briefly, plastic-adherent cells of PBLs were cultured in the presence of recombinant human granulocyte/macrophage-colony stimulating factor (800 units/ml; Kirin Brewery Co., Tokyo, Japan) and IL-4 (500 units/ml; Genzyme, Miami, FL) for 4 days. The cultured DCs were then pulsed with α-GalCer (100 ng/ml) or control vehicle for 12 h. Nonadherent cells were washed extensively, irradiated (5000 rads), and cocultured for 72 h with murine Vα14 NKT cells from RAG-1−/− Vα14+ Vβ8.2+ mice (3). The ability of DCs from melanoma patients to present α-GalCer was evaluated by [3H]thymidine uptake of the murine Vα14 NKT cells.

Results

Evaluation of the in Vitro and in Vivo Cytotoxic Activity of α-GalCer-activated Vα24 NKT Cells. Human Vα24 NKT cells purified from umbilical cord blood were cultured with α-GalCer and IL-2 for 14 days (7), and their cytotoxic activity against tumor cells was assessed. As shown in Fig. 1A, >87% of the cultured cells were found to express the invariant Vα24/Vβ11 NKT cell antigen receptor. These cells displayed a potent cytotoxic activity against Daudi lymphoma (Fig. 1B, left), which was inhibited by concanamycin A, suggesting a perforin-dependent killing mechanism (Fig. 1B, right).

Moreover, we demonstrated that a variety of human tumor cell lines were susceptible to the activated Vα24 NKT cells. As shown in Fig. 24, K562, Daudi, and Molt-4 (hematopoietic tumors) were highly

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The abbreviations used are: α-GalCer, α-galactosylceramide; NK, natural killer; PBL, peripheral blood lymphocyte; DC, dendritic cell; APC, antigen-presenting cell; IL, interleukin; Con A, concanavalin A.
susceptible to the activated Vα24 NKT cells. Similar susceptibility was shown by SK-Mel-28 and HMV-1 (malignant melanoma cell lines); PC10, PC6, PC13, and ABC-1 (lung cancer cell lines); RCM-1 (colon cancer line); TN-1 (neuroblastoma cell line); and HeLa cell lines were also considerably susceptible. On the other hand, certain cell lines, such as PANC-1 (pancreas cancer), Alexander hepatoma, and SHIN-3 (ovarian cancer), appeared to be relatively resistant. Con A blasts of normal PBLs were not susceptible by the activated Vα24 NKT cells (Fig. 2A, bottom, right). We found no correlation between the levels of surface expression of class I MHC molecules on the tumor cells (Fig. 2B) and the levels of cytotoxicity (Fig. 2A). Thus, the effector mechanisms of the activated Vα24 NKT cells cannot be explained simply as a conventional NK cell killing.

To further investigate the in vivo function of α-GalCer-activated Vα24 NKT cells, we carried out a Winn’s assay (8). As shown in Fig. 3, the tumor growth was dramatically inhibited if T.Tn tumor cells were inoculated together with activated Vα24 NKT cells, suggesting cytotoxic activity of α-GalCer-activated Vα24 NKT cells in vivo.

**Activation of Vα24 NKT Cells Obtained from Patients with Malignant Melanoma.** In view of several reports suggesting an impairment of the cytotoxic activity of T or NK cells in tumor-bearing patients (12–14), we investigated whether the Vα24 NKT cells from tumor patients could be activated to exhibit cytotoxic activity. Our results demonstrated that the number of Vα24 NKT cells in PBLs from 13 patients with malignant melanomas was significantly low compared with those found in umbilical cord bloods or in PBLs from adult individuals (Fig. 4A). However, the Vα24 NKT cells in patients responded well to α-GalCer, and their numbers increased greatly during the 14-day culture (Fig. 4B). This increase in their cell number varied between 119 and 1449 times, which is as good as the expansion in healthy individuals (data not shown). In addition, the activated Vα24 NKT cells from the patients were revealed to have cytotoxicity against various tumor cells, such as Daudi lymphoma and SK-Mel-28 melanoma cells (Fig. 4C). Therefore, Vα24 NKT cells in tumor patients appeared to be functionally normal in terms of ligand-induced activation, expansion, and antitumor cytolytic activity.
Efficient Antigen Presentation by DCs from Melanoma Patients. Finally, we addressed the question whether DCs from melanoma patients preserved their capacity to present α-GalCer to stimulate NKT cells, because it is important to evaluate patient’s DC functions for practical reasons before immunotherapy. For this purpose and based on the well-known capacity of human DCs to stimulate murine Vα14 NKT cells (5), murine Vα14 NKT cells as an indicator were stimulated by α-GalCer-pulsed DCs obtained from a patient. As shown in Table 1, stimulation indexes obtained by patient DCs were in the range of 14–74, which were similar to those observed by healthy volunteers. Therefore, α-GalCer-pulsed DCs obtained from malignant melanoma patients as well as those from healthy donors activated murine Vα14 NKT cells equally well to induce significant proliferative responses.

Discussion

Here, we demonstrate that freshly isolated Vα24 NKT cells from PBLs of tumor-bearing patients or healthy donors displayed perforin-dependent antitumor cytotoxicity in vitro (Figs. 1, 2, and 4) and in vivo (Fig. 3), after activation with α-GalCer. This cytotoxic activity was not dependent on the expression level of MHC class I molecules on the target cells, suggesting the effector mechanisms of Vα24 NKT cells are different from those observed in conventional NK cells.

The α-GalCer/CD1d-NKT system reveals to be a promising new approach for immunotherapy aimed at treatment of human cancers for the following reasons: (a) the level of cytotoxicity of activated Vα24 NKT cells is very high and effective against a wide variety of tumor cells (Fig. 2); (b) normal cells are not susceptible to the activated Vα24 NKT cells; (c) the activation of Vα24 NKT cells by α-GalCer is totally dependent on a CD1d molecule, which is monomorphic among individuals (15), indicating that α-GalCer can be applied to all patients, irrelevant to MHC haplotypes; (d) Vα24 NKT cells are functionally normal, even in the tumor-bearing status, although the initial numbers of Vα24 NKT cells in these patients were significantly lower than those found in healthy volunteers; (e) the antigen-presenting functions of DCs in cancer patients can be evaluated before immunotherapy by the assay system using mouse Vα14 NKT cells as an indicator.

Extensive efforts have been made for the detection and isolation of the peptides bound to classical MHC molecules, which can be used as tumor vaccine (16, 17). In fact, some tumor antigens have been introduced to clinical trials with significant effects (16, 17). However, there are several theoretical and practical problems. Among others, one of the most critical is that the expression of MHC class I molecules on tumor cells in the tumor nests is quite variable (18, 19). Indeed, MHC low-expressing tumor cells are found to escape from the immunotherapy (20). Another important problem is that MHC molecules are polymorphic; therefore, a peptide isolated from one patient with a certain MHC haplotype is likely to be irrelevant for other cancer patients with different MHC haplotypes. From an immunotherapy viewpoint, the Vα24 NKT cell system appears to have distinct advantages over other antitumor mechanisms. For example, CD1d is monomorphic among species, and human NKT cells are highly effective against a wide variety of tumor cells expressing low levels of MHC in an NK-like mechanism. Therefore, some important problems raised in the CTL/MHC peptide therapy appear to be resolved. Thus, the combination of NKT cell and

Table 1. Ability of patient’s DCs to present α-GalCer

| Age | Sex | Stage | α-GalCer cpm | Vehicle cpm | Index
|-----|-----|-------|-------------|-------------|-------
| Healthy volunteers |     |       |             |             |       |
| 33 M | M | I | 85290 ± 3904 | 3478 ± 703 | 24.5 |
| 34 M | M | I | 92403 ± 1420 | 3611 ± 895 | 25.6 |
| Melanoma patients |     |       |             |             |       |
| 70 M | M | II | 75116 ± 5662 | 1146 ± 331 | 65.5 |
| 68 M | M | III | 84535 ± 289 | 3088 ± 850 | 27.4 |
| 67 M | M | III | 55014 ± 2941 | 2804 ± 673 | 18.7 |
| 79 F | F | III | 72399 ± 4970 | 2435 ± 730 | 29.7 |
| 78 F | F | III | 75711 ± 8787 | 5276 ± 1560 | 14.4 |
| 25 F | F | IV | 66228 ± 1732 | 890 ± 67 | 74.3 |

a Murine Vα14 NKT cells (2 × 10^5 cells/well) were cultured with patient’s DCs (5 × 10^5 cells/well) pulsed with α-GalCer or vehicle.

b The clinical stages of each patients are indicated according to the international Tumor-Node-Metastasis (TNM) classification.

c The stimulation index was calculated from the following equation: cpm of α-GalCer-treated group/cpm of vehicle-treated group.

d All patients received chemotherapy with dacarbazine, nimustine, and vincristine combined with topical administrations of IFN-β.  

Fig. 3. Inhibition of tumor growth in vivo by α-GalCer-activated Vα24 NKT cells. A human esophageal cancer T.Tn cells (1 × 10^7) were s.c. inoculated into the back of BALB/c nude mice with ( Curtis) or without (○) activated Vα24 NKT cells (1 × 10^7). Bars, SD. B, photographic view of tumors. Two representative mice with or without Vα24 NKT cells are shown. Three independent experiments with a total of five mice in each group showed similar results.

Fig. 4. Vα24 NKT cells from patients with melanomas. A, numbers of Vα24 NKT cells in umbilical cord blood (n = 21), PBLs of adults (n = 15), and PBLs of melanoma patients (n = 13) were calculated by flow cytometric analysis. The results are depicted as mean values; bars, SD. Statistical analysis was performed with the Mann-Whitney U test. *P < 0.0001; **P < 0.01. B, expansion of Vα24 NKT cells upon stimulation with α-GalCer. PBLs from melanoma patients (n = 3) were cultured by the methods as described in Fig. 1. The absolute numbers of Vα24/NK11 NKT cells were calculated by using flow cytometric analysis. Each symbol represents a single individual patient. C, cytotoxic activity of the activated Vα24 NKT cells was assessed by 51Cr release cytotoxic assay on Daudi lymphoma and SK-Mel-28 melanoma cells. Bars, SD.
CTL/MHC peptide therapy appears to be a feasible and an approach worth developing, an approach that may become efficient and effective tools for the eradication of human cancers.

In addition, it is particularly interesting to note that the murine Vα14 NKT cells can be used as an indicator to evaluate functions of patient DCs. It is important to know the functional ability of immune systems in patients before immunotherapy, because patient immune functions, including antigen-presenting activity and/or lymphocyte functions, can be impaired (12–14). Despite the above assumptions, the data shown in Table 1 have demonstrated clearly that human DCs from melanoma patients as well as healthy volunteers activate murine Vα14 NKT cells with α-GalCer effectively. Because stimulation indexes obtained by DCs of patients are in a similar range to those of healthy individuals, we speculate that most melanoma patients with different clinical stages have normal DC functions. In addition, proliferative responses and cytolytic functions of Vα from melanoma patients as well as healthy volunteers activate murine Vα14 NKT cells with α-GalCer effectively. Thus, by the experimental work.

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