Dendritic cells pulsed with alpha-galactosylceramide induce anti-tumor immunity against pancreatic cancer in vivo

S. Nagaraj1,2*, C. Ziske1*, J. Strehl1, D. Messmer3, T. Sauerbruch1 and I. G. H. Schmidt-Wolf1

1Department of Internal Medicine I, Rheinische Friedrich-Wilhelms-Universität, Sigmund Freud Strasse 25, 53105 Bonn, Germany
2H. Lee Moffitt Cancer Center, University of South Florida, MRC-2 E, Room 2068, 12902 Magnolia Dr., Tampa, FL 33612, USA
3Department of Medicine, John and Rebecca Moores Cancer Center, University of California San Diego, La Jolla, CA 92093-0663, USA

Keywords: alpha-galactosylceramide, dendritic cells, NKT cells, pancreatic cancer

Abstract

Ductal pancreatic adenocarcinoma is the fourth leading cause of cancer death in the Western world. Unfortunately, recent advances in diagnostics, staging and therapy have not resulted in significant improvements. Thus, new approaches are necessary to improve the outcome of patients with exocrine pancreatic cancer. We tested triggering of specific T lymphocytes in vivo by using the immunocompetent mouse strain C57BL/6. In the present study, we tried to enhance the anti-tumor effect against pancreatic carcinoma by supplementary triggering of NKT cells in vivo. We challenged Panc02 tumor-bearing mice by intratumoral vaccination with alpha-galactosylceramide (alpha-GalCer)-loaded dendritic cells (DCs). A significant expansion of IFNγ-producing NKT cells was observed which also correlated with decrease in tumor growth in vivo. Hence, DCs loaded with alpha-GalCer could lead to a novel treatment option for patients with pancreatic cancer.

Introduction

Ductal pancreatic adenocarcinoma is the fourth leading cause of cancer death in the Western world. Unfortunately, intense research in diagnostics, staging and therapy has not resulted in a significant improvement of survival. Thus, new approaches are necessary to improve (advance) the outcome of patients with exocrine pancreatic cancer. We have previously shown that co-culturing of NKT cells with dendritic cells (DCs) transfected with pancreatic tumor cell line-derived RNA reverses pancreatic carcinoma cell resistance by directly triggering NKT lymphocytes in vitro (1). Furthermore, we tested triggering of specific T lymphocytes in vivo by using an immunocompetent mouse strain (C57BL/6). In the present study, we investigated to what extent the anti-tumor effect against pancreatic carcinoma can be enhanced by supplementary triggering of NKT cells. Previously, it was shown that NKT lymphocytes mediate a rapid reaction to the glycolipid drug alpha-galactosylceramide (alpha-GalCer), which triggers release of large amounts of cytokines into the serum within 12 h (2). Alpha-GalCer is presented by a monomorphic CD1d on DCs and recognized by the invariant Vα14 TCR on murine NKT cells (3, 4). Administration of alpha-GalCer to murine DCs led to a prolonged response (>4 days) and a large expansion of IFNγ-producing NKT cells as well as greater resistance to metastases of a B16 melanoma (2). Therefore, we investigated the use of alpha-GalCer-loaded DCs for tumor growth control of pancreatic cancer in a murine model.

Methods

Mice

Male C57BL/6 (H-2b) mice 6- to 10-week olds were purchased from Charles River Inc. (Sulzfeld, Germany). Animals were kept in a pathogen-free environment approved by the Institutional Animal Care and Use Committee, and all experiments were conducted in accordance with the principles and procedures approved by the local regulatory agency.

Cell lines and culture medium

Panc02 is a tumorigenic murine pancreatic cell line with ductal morphology. It was derived in 1984 from...
a methylcholanthrene-induced tumor growing in a C57BL/6 female mouse. It is a ductal adenocarcinoma of the pancreas, which has been shown to produce rapidly growing local tumors following subcutaneous inoculation, which are highly resistant to anti-tumor agent. The cell line is tumorigenic after injection of only $10^3$ pancreatic cells into nude or immunocompetent C57BL/6 mice and leads to death from progressive disease of all treated animals within a few weeks. Panc02 cells were maintained in RPMI cell culture medium (GIBCO/BRL, Berlin, Germany), supplemented with 10% FCS (PAA, Cöbe, Germany), 100 U ml$^{-1}$ penicillin and 100 µg ml$^{-1}$ streptomycin (Seromed, Jülich, Germany) and incubated at 37°C under 5% CO$_2$. Cells were made non-adherent by brief trypsination with 0.025% trypsin.

mAbs and flow cytometry

Two-color immunostaining was performed with FITC- and PE-conjugated mAbs. The following anti-mouse mAbs and appropriate isotype controls were used: MHCI (I-A), CD11c, CD14, CD40, CD80, CD83 and CD86 (all from PharMingen, BD, Hamburg, Germany).

Generation of bone marrow-derived DC

DCs were prepared as described by Inaba et al. (5) with minor modifications. Bone marrow cells were flushed from mouse tibia and femur, depleted from erythrocytes by incubating in 0.9% ammonium chloride for 3 min at 37°C and depleted of T and B lymphocytes and granulocytes by incubation (20 min, 4°C) with rat anti-mouse CD4, CD8, Ly6G and CD45R mAbs (Serotec, Düsseldorf, Germany) followed by incubation (15 min, 4°C) with goat anti-rat IgG conjugated to magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Labeled cells were then separated in a magnetic field using MACS columns. Unbound cells were cultured in DC medium consisting of RPMI 1640 medium containing 10% FCS, 1% glutamine and antibiotics supplemented with 750 U ml$^{-1}$ granulocyte macrophage colony-stimulating factor, 500 U ml$^{-1}$ IL-4 (Biozol, Eching, Germany) and on day 7 with 0.1 µg ml$^{-1}$ LPS (Sigma, Munich, Germany). Expression of CD11c, MHCI, CD40, CD80 and CD86 was quantified by EPICS Profile II flow cytometer (days 1, 3, 5 and 7).

Pulsing DCs with alpha-GalCer

On day 6, 100 ng ml$^{-1}$ of alpha-GalCer (Kirin Breweries, Tokyo, Japan) was added to immature bone marrow DCs for 40 h. Mature alpha-GalCer-pulsed DCs were collected on day 8.

In vivo tumor inoculation and immunization

Tumor was inoculated subcutaneously at day +7. Briefly, Panc02 tumor cells were injected into the right flank of the mice at $1 \times 10^5$ in 0.02 ml of PBS. Throughout these procedures aseptic conditions were maintained. Mice were pre-vaccinated on day 0 subcutaneously and post-vaccinated subcutaneously on days +14 and +28 with $6 \times 10^5$ DC pulsed with alpha-GalCer in 100 µl PBS. A series of five animals have been processed with the above-mentioned procedure and each animal was individually examined on alternate days for measurement of tumor size. The volume of the tumor was periodically calculated by using the formula $V = 0.5a \times b^2$, where a and b are the long and short diameters, respectively, measured by a slide caliper. Animals were sacrificed when the tumor reached a volume set prior to therapy according to the local animal committee requirements, or when they became moribund prior to the expected tumor volume.

Generation of murine splenocytes

Spleens from mice were isolated under sterile conditions. Single-cell suspensions were prepared and red cells removed from spleens by incubating in 0.9% ammonium chloride for 3 min at 37°C.

IFN$\gamma$ secretion by splenocytes

An ELISA for IFN$\gamma$ with matched antibody pairs was performed according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). In brief, 96-well filtration plate (Millipore, Schwalbach, Germany) was coated with rat anti-mouse IFN$\gamma$ capture antibody (R&D Systems). Spleen cells ($2 \times 10^5$) from immunized mice were cultured with or without alpha-GalCer for 72 h. The plates are washed and incubated with biotinylated anti-mouse IFN$\gamma$ detection antibody. Spots were developed by addition of alkaline phosphatase conjugated to streptavidin and subsequently coloring by BCIP/NBT stain (St Louis, MO, USA).

Statistical analysis

Results from quantitative studies were expressed as the mean ± SD. Statistical comparison within and between various treatments were made by analysis of variance for repeated measures, followed by Fisher's least significant difference tests of repeated measures when appropriate. A value of $P < 0.05$ was accepted as the level of significance. All computations have been performed with the Statistical Package for Social Sciences (SPSS), version 10.0 (SPSS Inc., Chicago, USA).

Results

DCs

DCs were obtained by flushing bone marrow cells from the femurs and tibias of mice and then progenitor cells were enriched by depletion of T cells, B cells, macrophages, granulocytes and erythrocytes as described earlier. Flow cytometry on day 7 of cultivation showed expression of markers typical of DC (MHCI, CD11c, CD40, CD80, CD83 and CD86; data not shown). There was no difference between pulsed and unpulsed DCs.

IFN$\gamma$ production by splenocytes

After 72 h of splenocyte re-stimulation with or without alpha-GalCer, IFN$\gamma$ secretion of splenocytes was measured by an enzyme-linked immunospot assay (Fig. 1). Re-stimulated splenocytes from mice immunized with alpha-GalCer-pulsed DCs produced significantly ($P = 0.005$) higher number of IFN$\gamma$ spots as compared with splenocytes from mice immunized with unpulsed DCs or alpha-GalCer alone. Even without in vitro
re-stimulation of the splenocytes, an increase of IFN-γ-producing cells is detectable after immunization of mice with alpha-GalCer-pulsed DCs.

**Induction of anti-tumor responses: in vivo tumor challenge model**

To assess the anti-tumor activity of alpha-GalCer, mice were pre-vaccinated subcutaneously on day 0 with PBS only or with alpha-GalCer-pulsed DCs, and challenged by injecting $1 \times 10^5$ Panc02 cells on day +7. On days +21 and +28, mice were post-vaccinated (PBS only or DCs pulsed with alpha-GalCer, respectively). Immunization of mice with DCs pulsed with alpha-GalCer prevented tumor growth until week 4 and strongly decreased tumor growth in comparison to the control group ($P = 0.005$; Fig. 2). This was demonstrated by a decrease in tumor volume (Fig. 2) as well as by an increase in the percentage of tumor-free mice (Fig. 2). In addition, survival time was prolonged by the use of alpha-GalCer-pulsed DCs. In the treatment group, 50% of animals were dead after 5.2 weeks as compared with 2.5 weeks in the control group ($P < 0.05$; Fig. 2).

**Discussion**

New approaches are necessary to improve the outcome of patients with exocrine pancreatic cancer. DCs pulsed with peptides are more potent mediators of the lyses of autologous and allogeneic cancer cells *in vitro* as compared with DCs alone. Studies have proved that antigen-loaded DCs are potential activators of anti-tumor responses (6–19).

In some cancers like pancreatic carcinoma, the use of allogeneic tumor cells as a source of antigens has been strongly suggested (20). However, today even though >200 tumor-associated epitopes are recognized by T cells, most of them do not elicit a strong immune response (21).

Pulsing of DCs with small peptides is the easiest method of delivering antigens to DCs. Another clinical study used monocyte-derived DCs pulsed with idiomorphic peptide derived from B cell lymphoma (22). A variety of clinical studies have used peptide-pulsed DCs (23).

The efficacy of peptide-loaded DC vaccination in terms of CTL induction and anti-tumor activity depends on additional critical factors, such as the route of DC administration and the origin of the DCs. Recent results with tumor-associated antigens peptide-loaded murine bone marrow-derived DCs indicated that subcutaneously injected DCs had greater anti-tumor activity than intravenously injected DCs. Subcutaneously injected DCs home to T cell areas of the draining lymph nodes, whereas intravenously injected DCs home to the spleen (24). Although in mice the induced immunity was influenced by the route of administration of the vaccine, the route of administration did not make a difference in cancer patients (25). Murine models have also provided evidence that antigen-presenting cells from tumor-bearing animals may be
DCs pulsed with α-GalCer induce anti-tumor immunity

less effective in inducing anti-tumor responses than DCs from cancer-free normal mice (26).

Mayordomo et al. showed that mice injected with antigen-loaded DCs were protected against subsequent challenge with the same tumor (11). Even in a therapeutic setting, such a vaccination was effective since tumors regressed in treated animals (9).

Alpha-GalCer is presented by a monomorphic CD1d on DCs and recognized by the invariant Vα14 TCR on murine NKT cells (3, 4). Naïve subcutaneous injection of alpha-GalCer in vivo induces anti-tumor activity against metastatic tumors macroscopically (4) and microscopically (27), indicating that it takes a longer time for DCs to present alpha-GalCer, to activate Vα14 NKT cells and to induce anti-tumor activities in vivo (28). In concordance to published studies, our results show that alpha-GalCer-pulsed DCs are more effective than the direct injection of alpha-GalCer (2, 28).

NKT cells express TCR and CD1D molecules recognize this TCR. Alpha-GalCer is a synthetic derivate of CD1D molecule and binds strongly to the TCR. To show the proof of concept that there is strong response by NKT cells and some NK cells due to their interaction with DCs loaded with alpha-GalCer, inducing IFNγ production, we injected mice intravenously with DCs pulsed with alpha-GalCer or without alpha-GalCer only. On re-stimulation with alpha-GalCer, splenocytes produced significant higher levels of IFNγ when they were injected with DCs pulsed with alpha-GalCer than with alpha-GalCer only (P = 0.004) (Fig. 1). These results are similar to the findings of Fuji et al. (2). However, the results presented in Fig. 1 do not necessarily show that NKT cells are expanded by the injection of alpha-GalCer-pulsed DCs. Stimulation of NKT cells with alpha-GalCer is known to induce expansion of other cells including NK cells and some T cells. Further experiments have to be performed to determine which cells are expanded and produce the cytokine.

In vivo injection of alpha-GalCer (29) or IL-12 (30) prevents liver or lung metastasis of tumors, malignant tumor cells, including LLC (30), Colon 26 or a TAP-2-deficient mutant of Rauscher virus-induced lymphoma (RMA-S) (29) via activation of Vα14 NKT cells. Its therapeutic effect can be traced back to its ability to bind CD1d and activate invariant NKT cells, which results in the production of T\(_{\text{H}}\)1 and T\(_{\text{H}}\)2 cytokines (31–33).

In this paper, we investigated the role of alpha-GalCer-pulsed DCs and its effect on pancreatic tumors. In the present study, mice were pre-vaccinated once, challenged with Panc02 tumor cells subcutaneously and vaccinated twice and the tumor sizes were measured. The tumors reached an average volume of 212 mm\(^3\) after 7 weeks compared with 3240 mm\(^3\) of the control group P < 0.005 (Fig. 2). Smyth et al. (34) showed that transfer of alpha-GalCer-pulsed DCs followed by systemic IL-21 caused an even more significant reduction in established (day 8) metastatic burden and prolonged survival.

Panc02 is a tumorigenic murine pancreatic cell line with ductal morphology. It is a ductal adenocarcinoma of the pancreas, which has been shown to produce rapidly growing local tumors following subcutaneous inoculation, which are highly resistant to anti-tumor agent. The cell line is tumorigenic after injection of only 10\(^3\) pancreatic cells into nude or immunocompetent C57BL/6 mice and leads to death from progressive disease of all treated animals within a few weeks. The tumor model has already been used by various groups either subcutaneously or orthotopically (1, 35, 36). Of course, results from the subcutaneous model are limited. Further experiments should be done in the orthotopic model which is closely related to the situation in humans.

For application of tumor peptide therapy to cancer patients, MHC haplotypes have to be considered. Moreover, the MHC level is reported to be different in the region of tumor tissues (37, 38), and thus, all tumor cells cannot be targets of CTL.

Therefore, NKT cell activation via alpha-GalCer appears to be ideal to circumvent these problems as shown in our experiments. Our findings suggest the potential utility of alpha-GalCer-pulsed DCs for cancer immunotherapy in pancreatic cancer patients inducing a strong anti-tumor immune response.

Acknowledgements

We are grateful to Kirin Breweries, Tokyo, Japan for their kind gift of alpha-GalCer (KR7N00). We are thankful to Mazzolini, University de Navarra, Pamplona, Spain for kindly providing Panc02 cell line. This work was kindly supported by a generous grant of the Deutsche Krebshilfe, Bonn, Germany.

Abbreviations

Alpha- \(\alpha\)-galactosylceramide

GalCer \(\alpha\)-galactosylceramide

DC \(\alpha\) dendritic cell

References


