Cancer Letters xxx (2017) 1–9

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Co-delivery of tumor-derived exosomes with alpha-galactosylceramide on dendritic cell-based immunotherapy for glioblastoma

Hongyu Liu [a, 1], Ling Chen [a, 1], Jialin Liu [a, 1], Hengxing Meng [a], Rong Zhang [b], Lin Ma [c], Liangliang Wu [d], Songyan Yu [e], Fei Shi [f], Ying Li [c], Lijun Zhang [d], Lingxiong Wang [d], Shiyu Feng [a], Qi Zhang [e], Yaojun Peng [d], Qiyan Wu [d], Chunxi Liu [d], Xin Chang [g], Lin Yang [a], Xinguang Yu [a, **, 2], Tianyi Liu [d, *, 2]

[a] Department of Neurosurgery, Chinese PLA General Hospital, Beijing 100853, China
[b] Division of Cancer Immunotherapy, Exploratory Oncology Research & Clinical Trial Center, National Cancer Center, Chiba 277-8577, Japan
[c] Department of Radiology, Chinese PLA General Hospital, Beijing 100853, China
[d] Key Laboratory of Cancer Center, Chinese PLA General Hospital, Beijing 100853, China
[e] Department of Endocrinology, Chinese PLA General Hospital, Beijing 100853, China
[f] Department of Skull Base Surgery Center, Otorhinolaryngology Head and Neck Surgery, Xuanwu Hospital, Capital Medical University, Beijing 100053, China
[g] Department of Clinical Laboratory, Weihai Municipal Hospital, Weihai 264200, China

ARTICLE INFO

Article history:
Received 4 May 2017
Received in revised form
12 September 2017
Accepted 16 September 2017

Keywords:
Glioblastoma multiforme
Exosome
Dendritic cells
Invariant natural killer T cells
Immunotherapy

ABSTRACT

Dendritic cell (DC) vaccine–based immunotherapy for glioblastoma multiforme (GBM) has shown apparent benefit in animal experiments and early-phase clinical trials, but the survival benefit is variable. In this work, we analyzed the mechanism of the potent antitumor immune response induced in vivo by tumor-associated antigen (TAA)-specific DCs with an invariant natural killer T cell (iNKT) adjuvant in orthotopic glioblastoma-bearing rats vaccinated with tumor-derived exosomes and α-galactosylceramide (α-GalCer) -pulsed DCs. Compared with traditional tumor lysate, exosomes were utilized as a more potent antigen to load DCs. iNKTs, as an effective cellular adjuvant activated by α-GalCer, strengthened TAA presentation through their interaction with DCs. Co-delivery of tumor-derived exosomes with α-GalCer on a DC-based vaccine showed powerful effects in glioblastoma immunotherapy. This vaccine induced strong activation and proliferation of tumor-specific cytotoxic T lymphocytes, synergistically breaking the immune tolerance and improving the immunosuppressive environment.

Introduction

Glioblastoma multiforme (GBM) is a highly malignant primary brain tumor associated with an extremely poor prognosis and median survival of only 8–12 months [1–4]. The current standard treatment of GBM is complete surgical resection with temozolomide adjuvant chemotherapy and radiation. Even with this treatment, the 5-year survival rate is less than 10% [5]. Although several novel agents like bevacizumab recently gained approval in the U.S. and demonstrated clinical benefit in GBM, there was no improvement in overall survival [6–8]. Therefore, an improved treatment for GBM is urgently needed.

Dendritic cells (DCs) are the most powerful antigen-presenting cells [9]. DCs activate and stimulate the proliferation of T and B cells via their strong capacity to catch, process and submit antigens, and then induce an immune response. Based on this capacity, there have been many immunotherapy studies of DC vaccines [10-14]. In recent years, tumor lysates have been used as a source of antigens for DC vaccines. These vaccines exhibited some effectiveness in glioblastoma treatment, although the response rate was limited [15–17].

* Corresponding author.
** Corresponding author.
E-mail addresses: xinguang_yu@263.net (X. Yu), tianyiliu08@hotmail.com (T. Liu).
[1] Co-first authors: Hongyu Liu, Ling Chen and Jialin Liu.
[2] Co-senior authors: Tianyi Liu and Xinguang Yu.

https://doi.org/10.1016/j.canlet.2017.09.022
0304-3835/

In 1983, Johnstone et al. termed a small vesicle first detected in sheep reticulocytes [18] the exosome [19]. Exosomes are 30-to-100-nm vesicles secreted by almost all types of cells, including tumor cells [20,21]. These vesicles contain proteins and nucleic acids that are associated with donor cells [22]. Exosomes also have specific membrane markers, such as tetraspanins (e.g., CD9, CD63, and CD81), heat-shock protein (Hsp70), major histocompatibility complex molecules (MHC I and II), and co-stimulatory molecules [23]. In 1996, Raposo et al. reported that B cell-derived exosomes induced antigen-specific MHC class II restricted T cell responses [24]. Later, Zitvogel et al. demonstrated that DC-derived exosomes pulsed with tumor-derived peptides elicited a potent antitumor T cell response in tumor-bearing mice [25]. These results indicated that exosomes have the potential for cancer immunotherapy.

In 2001, Wolfers et al. determined that tumor-derived exosomes could be used as a source of shared tumor rejection antigens for cytotoxic T lymphocyte (CTL) cross-priming [23]. Based on this finding, many tumor-based immunotherapy studies have been conducted in cancers such as melanoma, glioma, hepatocellular carcinoma, and renal cell carcinoma [10–14]. These studies demonstrated that the effect of tumor-derived exosomes in inducing antigen-specific CTL responses was better than that of tumor lysates.

The therapeutic immunity elicited by DC vaccines can be strengthened by pulsing with some agents. Invariant natural killer T cells (iNKTs) are a special subset of T cells that has the combined characteristics of T and NK cells [26]. iNKTs express uniform activated T cell receptors and proliferate via recognizing a specific glycolipid antigen, α-galactosylceramide (α-GalCer), bound to CD1d [27]. As cellular adjuvants, activated iNKTs enhance innate and adaptive immune responses [28–30]. For example, recent studies demonstrated that the potent synthetic iNKT agonist, α-GalCer, combined with DC vaccines, enhanced conventional T cell responses by upregulating immunostimulatory factors such as CD40 by encouraging iNKT:DC interactions. This vaccine was highly effective as immunotherapy for glioma [31]. In some animal studies, co-delivery of tumor antigens and α-GalCer applied to anticancer vaccines was highly effective in inducing long-lasting immunity by activating iNKT cells [32–35]. A significant immunotherapeutic effect of α-GalCer-pulsed DCs has been also detected in breast cancer, melanoma, and glioma [31,36–37].

In the current study, we investigated the specific anti-tumor immune response induced by co-delivery of tumor-derived exosomes with α-GalCer on DC-based immunotherapy for glioblastoma. The accumulated evidence can contribute to establishing a specific broad spectrum immunotherapeutic approach.

Material and methods

Cell line and animals

The rat C6 glioma cell line was purchased from the Chinese XieHe Medicine Institute (Beijing, China). 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) were centrifuged with 10^5 g 4 °C for 70 min. Supernatant divert into germ-free tubes, passed through a 0.22 μm filter to remove the exosomes and stored at −20 °C until to use. C6 cells were cultured in Dulbecco's modified Eagle's medium containing exosome-free 10% FBS. Bone marrow dendritic cells (BMDCs) were extracted from six-to-eight-week-old male Sprague Dawley rats (170 ± 10 g). The rat glioma model was established with adult male Sprague Dawley rats (270 ± 10 g). Spleens were harvested from six-to-eight-week-old male Wistar rats (180 ± 10 g) and were used for Mixed Lymphocyte Reaction (MLR) analysis. All rats were purchased from Vital River (Beijing, China) and were kept in specific pathogen-free conditions.

Exosome and lysate preparation

Exosomes were isolated using the exoRNeasy Serum/Plasma Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, filtered culture supernatant from C6 cells was mixed 1:1 with 2× binding buffer and added to the exoEasy membrane affinity column to bind the exosome vesicles to the membrane. After centrifugation, the eluent was discarded and wash buffer was added to the column to remove non-specifically retained material. After a second centrifugation, the eluent was again discarded and exosomes attached to the spin column were eluted in XE buffer.

Tumor lysate was obtained by freezing C6 cells in liquid nitrogen for 3 min, immediately followed by thawing in a 56 °C water bath. This procedure was repeated five times in rapid succession. The lysate was centrifuged at 100 g for 10 min to remove cellular debris, then passed through a 0.2-μm filter. Protein concentrations of the tumor lysates and exosomes were determined with the Micro BCA protein assay kit (CWBIO, Beijing, China). Samples were stored at −20 °C until use.

Electron microscopy

The purified C6 cells-derived exosomes were applied to 400-meshcopper grids (EMS, Matfield, PA, USA), and negatively stained with 2% uranyl acetate at room temperature before examined with HT7700 electron microscope (Hitachi, Tokyo, Japan) at 100 kV.

Western blotting

Various amounts of protein prepared from C6 cell lysates and exosomes were loaded onto SDS-PAGE and transferred to a PVDF membranes. The primary antibodies used were: TSG101 (1:200), CD63 (1:200), CD9 (1:1000), GM130 (1:500), Primary antibodies were incubated overnight at 4 °C followed by washing and the application of secondary HRP-conjugated antibody. The immunoreactive bands were visualized with a chemiluminescent substrate. All antibodies were from Abcam (Cambridge, UK).

In vitro generation of immature rat BMDCs and loading DCs with tumor exosomes or lysate

Tibias and femurs were removed from 6-to-8-week-old rats. All muscle tissues were removed with sterile scissors. The bones were then washed twice with RPMI medium and were transferred into a dish containing fresh RPMI medium. Both ends of the bones were cut, and a 10-mL syringe with a 20-gauge needle was used to flush the bone marrow into a new dish. Cell clusters within the marrow suspension were dispersed by vigorous pipetting. Then, cells were passed through a 100-μm filter to remove small pieces of bone and debris. After centrifugation at 200 g for 5 min, red cells were lysed with 2 mL lysis buffer (BD Biosciences, Franklin Lakes, NJ, USA) for 10 min. After washing, cells were plated in six-well plates at a density of 1×10^6/mL in RPMI 1640 medium containing 2 mM L-glutamine (Thermo Fisher Scientific) supplemented with 10% exosome-free FBS (obtained by prior ultracentrifugation at 100,000 g for 70 min), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL recombinant rat granulocyte macrophage colony-stimulating factor, and 10 ng/mL recombinant rat interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA). Cultures were fed every second day; 50% of the medium was aspirated and replaced with an equal volume of fresh medium containing the cytokines. Nine days after initial plating, non- and semi-adherent cells were harvested and regarded as immature DCs. These cells were loaded with C6 cell-derived exosomes (40 μg/mL) or lysates (100 μg/mL) overnight. Then, 100 ng/mL α-GalCer was added to the DC culture to allow complete maturation while incubating for 24 h at 37 °C.

Flow cytometric analyses

BMDCs were removed from plates and centrifuged at 450 g for 5 min. The pelleted cells were washed once with RPMI medium, once with MACS buffer (PBS containing 2% FBS and 0.5 M EDTA), and once with 0.5 M EDTA-PBS, followed by incubation with purified mouse anti-rat CD32 to block the Fc receptor. Then, cells were incubated at 4 °C for 20 min with the following antibodies: CD103-Alexa Fluor 647, RT1B-PE, CD80-PE, CD86-PE, CD4-PE, CD11b/c-PE, mouse IgG1 κ isotype control-Alexa Fluor 647, mouse IgG2a isotype control-PE and mouse IgG1 κ isotype control-PE. All antibodies were purchased from BD Biosciences or BioLegend (San diego, USA). Cells were analyzed using the BD FACSCanto II (BD Biosciences) after washing with 0.5 M EDTA-PBS.

Mixed lymphocyte reaction

Spleens were harvested from Wistar rats under sterile conditions and homogenized mechanically into suspensions, followed by treatment with an erythrocyte lysing buffer (BD Biosciences). After centrifugation at 450 g, the cell pellet was resuspended in RPMI-1640 medium supplemented with 10% exosome-free FBS. Allogeneic T cells were isolated as responder cells using a nylon fiber column (Wako, Osaka, Japan) and adjusted to 1×10^6 cells/mL. Different numbers of DCs (5×10^4, 1×10^4, 2×10^3, and 1×10^3) were irradiated with X-rays (25 Gy) using an MBR 1520R-3 irradiator (Hitachi, Tokyo, Japan) and added to 96 well-plates containing allogeneic T cells. After 72 h of incubation, 20 μL MTT (5 mg/mL) were added to each well. The supernatant was removed before adding 150 μL dimethyl sulfoxide to dissolve the formazan crystals. Absorbance was measured at 490 nm.

Please cite this article in press as: H. Liu, et al., Co-delivery of tumor-derived exosomes with alpha-galactosylceramide on dendritic cell-based immunotherapy for glioblastoma, Cancer Letters (2017), https://doi.org/10.1016/j.canlet.2017.09.022