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ORIGINAL ARTICLE

IL-13Ra2- and glioma stem cell-pulsed dendritic cells induce glioma cell death in vitro

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Abstract

Objective Gliomas are the most common malignant tumors in the central nervous system. Despite multiple therapies including surgery, chemotherapy, and radiotherapy, the prognosis of patients remains poor. Immunotherapy is an alternative method of treating glioma, and the use of dendritic cell vaccines is one of the promising treatment options. However, there is no specific tumor cell antigen that can trigger dendritic cells (DCs). IL-13Ra2 is a specific antigen expressed in glioma cells; in the current study, we have attempted to explore whether IL-13Ra2 could be the antigen that triggers DCs and to envisage its application as potential therapy for glioma.

Methods The expression of IL-13Ra2 was detected in U251 glioma cell lines and primary glioma tissues using different methods. DCs from human blood were isolated and pulsed with recombinant IL-13Ra2, following which the cytotoxicity of these DCs on glioma cells was detected and analyzed.

Results About 55.9% human glioma tissue cells expressed IL-13Ra2, while normal brain tissue cells did not show any expression. DC vaccines loaded with IL-13Ra2, glioma cell antigen, and brain tumor stem cell (BTSC) antigen could significantly stimulate the proliferation of T lymphocytes and induce cell death in the glioma tissue. Compared to other groups, DC vaccines loaded with BTSC antigen showed the strongest ability to activate cytotoxic T lymphocytes (CTLs), while the glioma cell antigen group showed no significant difference.

Conclusion IL-13Ra2, which is expressed in gliomas and by glioma stem cells, as well as IL-13Ra2 could prove to be potential antigens for DC vaccine-based immunotherapy.

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Malignant gliomas are the most common and deadly brain tumors and arise from glial cells in the central nervous system (CNS) [1]. Gliomas are classified into four grades according to the histopathological criteria defined by the World Health Organization (WHO). Grade III and grade IV gliomas are the most aggressive types and are characterized by uncontrolled proliferation, necrosis, and infiltration [2]. Glioblastoma multiforme (GBM) is the most malignant type of glioma with a median survival time of 12 months despite an aggressive treatment consisting of surgery, radiotherapy, and chemotherapy. Substantial progress has been made in other malignant cancer therapies over the past decades; however, GBM remains essentially untreatable [3].

Apart from surgery, chemotherapy, and radiotherapy, immunotherapy is believed to be a promising method of treatment for malignant gliomas [4]. Tumor cells are recognized by immunocompetent cells that induce innate and adaptive immune responses. Adaptive immune responses are mostly characterized by tumor-specific T cells in the tumor microenvironment, and it has been demonstrated that these cell numbers are positively correlated with survival [5]. It is accepted that the brain is an immune-privileged organ that lacks dendritic cells (DCs) and T cells; however, recent studies have discovered lymphatic vessels in the brain and showed that tumor-derived antigens are transported to the cervical lymph nodes to stimulate specific T cells [6]. After amplification, these T cells are

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able to migrate back to the brain and kill tumor cells effectively. These findings indicate that immunotherapy could be a potential method for treatment of gliomas.

Dendritic cells (DCs) are the most powerful antigen presenting cells (APCs) [7]; we showed that immunotherapy using pulsed DCs from whole tumor extracts could significantly prolong the survival of GBM patients in a pilot clinical study [8]. However, by using the whole tumor extract, DCs do not get pulsed in all the patients. Hence, we attempted to find a more specific antigen expressed in gliomas to optimize the treatment.

Interleukin-13 receptor alpha 2 (IL-13Ra2) is a membrane receptor composed of 380 amino acids, belonging to the erythropoietin receptor family [9]. The gene encoding IL-13Ra2 is located on Xq24. Recent studies have demonstrated that IL-13Ra2 exhibits a highly tissue-specific expression in malignant tumors, especially in gliomas [10]. In addition, the expression is positively correlated with tumor malignancy, and no expression of IL-13Ra2 has been found in normal tissue and organs. Other researchers have found that IL-13Ra2 exhibits antigenicity and immunogenicity, which indicates its potential use in cancer immunotherapy [11].

Materials and methods

Human material

All material pertaining to human glioma and normal brain used in this study was obtained from the Department of Neurosurgery and Department of Neuropathology at Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, in accordance with the guidelines of the Ethical Committee.

Cell culture

Human glioma cell line U251 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 200 mM glutamine, 50 units/mL penicillin, 50 mg/mL streptomycin, and 10% FCS, as previously described [12].

Tumor tissue was enzymatically dissociated and cultured as floating neurospheres in serum-free DMEM/F-12 medium containing 20% BIT serum-free supplement (Stemcell Technologies Inc., Vancouver, Canada), EGF and bFGF at 20 ng/mL, respectively. Similarly, glioma U251 stem cells (U251-GSC), derived from the human glioma cell line U251, were cultured as non-adherent neurospheres in a serum-free supplemented medium as previously described. GSCs of more than six passages were used for further experiments. To induce GSC differentiation, we used the conventional serum-containing medium (10% FBS in DMEM) for seven days.

Peripheral blood (50 mL) was drawn from glioma patients; monocytes were isolated and cultured in RPMI-1640 medium supplemented with 10% patients' own serum. On the first, third, fifth and seventh day of culture, GM-CSF and Interleukin-4 were added to the culture medium. DCs were extracted and the samples were analyzed for purity by using flow cytometry, as previously described [8].

Clonal formation assay

Single-cell suspensions were calibrated to reach a concentration of 5,000 cells/mL in serum-free supplemented medium, diluted into gradient cell titers at 1,000, 500, 200, 100, 50, 20, and 10 cells/200, and further transferred into the wells of a 96-well microplate. To confirm the results obtained after the gradient dilution, more stringent clonal assays were performed by plating single cells into the 96-well plate. Clonal spheres (non-adherent, tight and spherical masses >75 μm in diameter) were counted under a microscope (Olympus CKX31, Tokyo, Japan) at the end of two weeks.

CCK-8 assay

Cells were seeded into 96-well plates at a density of 5×10^3 cells per well and maintained in culture medium for the indicated time. Thereafter, 10 μL CCK-8 reagent was added into the medium and incubated with the cells for 4 h. The optical density (OD) was measured at a wavelength of 450 nm using an ELISA plate reader, with subtraction of blank control reading. In addition, the morphological alterations of cells post-incubation were recorded microscopically.

ELISA

IFN-γ secretion was evaluated by detecting IFN-γ in the conditioned culture medium of DCs after indicated treatment for 48 h using a commercial IFN-γ ELISA kit (Uscn Life Science Inc., China) following the manufacturer's instructions.

Flow cytometry

Up to 106 cells were resuspended in the recommended buffer (containing PBS pH 7.2, 0.5% BSA, and 2 mM EDTA) with CD133/1 (AC133) antibody (1:11; Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at 4°C. Mouse IgG1 (1:11; Miltenyi Biotec) was used as the isotype control antibody. CD133 detection and analysis were performed on BD FACSAria [12].

Results

IL-13Ra2 was expressed in U251 glioma cells and human glioma tissue, not in normal brain tissue

The expression of IL-13Ra2 in U251 glioma cells and human glioma tissues was analyzed by immunostaining.

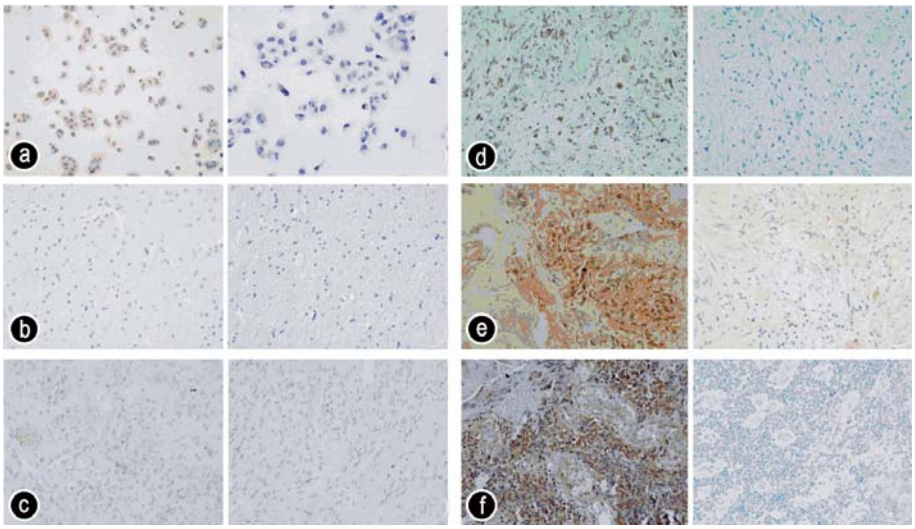

Fig. 1 (a) Expression of IL-13Ra2 in U251 cell line; (b) Expression of IL-13Ra2 in human normal brain tissue; (c) Expression of IL-13Ra2 in grade I gliomas; (d) Expression of IL-13Ra2 in grade II gliomas; (e) Expression of IL-13Ra2 in grade III gliomas; (f) Expression of IL-13Ra2 in glioblastomas

IL-13Ra2 was detected in U251 cells (Fig. 1a). In most of the human glioma tissues, no (or very faint) staining was observed in low-grade gliomas (grade I and grade II) (Fig. 1c, 1d), while in high-grade gliomas (grade III and grade IV), a strong IL-13Ra2 expression was observed (Fig. 1e, 1f). When the expression of IL-13Ra2 was evaluated in normal brain tissues, no signal was detected (Fig. 1b).

Expression of IL-13Ra2 was associated with glioma grades

We analyzed 59 gliomas tissues including 10 grade I gliomas, 17 grade II gliomas, 13 grade III gliomas, and 19 grade IV gliomas (Table 1). Five normal brain tissues were included as controls. The expression rates obtained are listed in Table 1. IL-13Ra2 was found to be significantly expressed in glioma tissues as compared to normal brain tissues (P = 0.0223), and high-grade gliomas expressed a level of IL-13Ra2 higher than that of low-grade gliomas (P = 0.0002) (Table 2). Although there is no difference in IL-13Ra2 expression between grade I and grade II (P = 0.1895), grade II and grade III (P = 0.1590), grade III and grade IV (P = 0.4012) gliomas, a significant difference was observed between grade I and grade III (P = 0.0097), grade I and grade IV (P = 0.002) and grade II and grade IV (P = 0.0140) gliomas. These data indicate that IL-13Ra2 is negatively correlated with glioma malignancy.

Growth patterns and identification of GSCs

GSCs were initially harvested using the neurosphere assay, with the GSCs growing as suspended spheres enriched with stemness characteristics. U251 glioma cells presented distinctive growth patterns under different culture conditions. The U251 cells showed adherence and had elongated branches (Fig. 2a); trypsinization for passage took ~4–5 min at 37 ℃. The U251 GSCs and human GSCs grew as floating spheres, which proliferated up to 100–200 μm in diameter within 3–4 days. The cells were mechanically filtered and dissociated into single cells without trypsinization, thereby forming secondary spheres for serial passage (Fig. 2b). CD133 was used as a marker and human GSCs showed more than 20% expression of CD133 (Fig. 2c), compared to less than 1% in glioma cells (Fig. 2d).

Table 1 Expression of IL-13Ra2 in gliomas of different grades

Tumor type	Total numbers	Positive	%
Grade I	10	1	10
Grade II	17	7	41.1
Grade III	13	9	69.2
Grade IV	19	16	84.2

Table 2 Expression of IL-13Ra2 in low-grade and high-grade gliomas

Tumor type	Total numbers	Positive	%	P
Low grade glioma	27	8	29.6	0.002
High grade glioma	32	5	78.1	

IL-13Ra2- and GSC-pulsed DCs induced T lymphocyte proliferation

Different effector to target cell ratios (E/T 1:5, 1:10, 1:20, and 1:40) were applied to identify the optimal ratio. IL-13Ra2- and GSC-pulsed DCs and T cells were co-cultured for 48 h and the proliferation of T cells was analyzed. In the group of GSC-pulsed DCs, E/T 1:10 induced the highest proliferation of T cells. However, in the group of IL-13Ra2-pulsed DCs, although DCs induced a very high proliferation of T cells, no difference was detected between different E/T subgroups. When DCs were not pulsed by any antigens, a very low rate of T cell proliferation was observed (Table 3). Hence, E/T 1:10 was considered the optimal E/T for T cell proliferation.

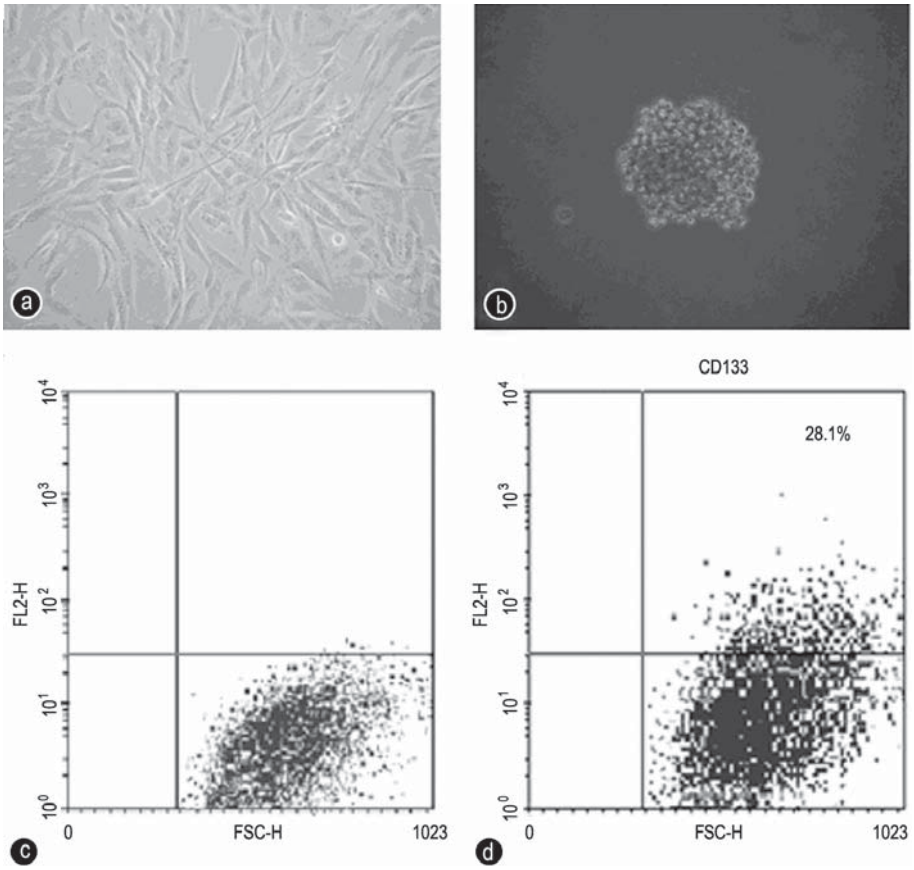

Fig. 2 (a) U251 cells; (b) Clonal formation assay in brain tumor stem cells (BTSCs); (c) Expression of CD133 in fourth generation BTSCs; (d) Expression of CD133 in fourth generation of glioma cells

Table 3 Ability to stimulate T lymphocyte proliferation in dendritic cell (DC) groups pulsed by different antigens

E/T	IL-13Ra2 pulsed DCs	GSCs pulsed DCs	Glioma cells antigen	Pure DCs
1:5	2.69 ± 0.19	3.41 ± 0.39	2.81 ± 0.24	1.11 ± 0.09
1:10	3.67 ± 0.27	4.01 ± 0.66	3.53 ± 0.41	1.44 ± 0.91
1:20	2.94 ± 0.24	3.78 ± 0.41	2.77 ± 0.33	0.91 ± 0.06
1:40	2.67 ± 0.21	3.43 ± 0.29	2.51 ± 0.19	0.69 ± 0.04

Pulsed DCs stimulated cytotoxic T cells to induce glioma cell death in vitro

T cells were stimulated by different groups of pulsed DCs as described. Pulsed DCs were further co-cultured with U251 cells or GSCs and cell death was analyzed. As shown in Table 4, the GSC-pulsed DCs group showed the highest rate of killing glioma cells in the U251 group, and the E/T of 20:1 showed the highest killing rate in both groups. However, in the group of GSCs, IL-13Ra2-pulsed DCs failed to show any effect when compared to the non-pulsed group, indicating that IL-13Ra2 was unable to pulse DCs to kill GSCs (Table 5). In both groups, using GSCs as the antigen showed the highest killing rate.

GSCs induced higher IFN- γ release on DCs

Since GSC-pulsed DCs showed the highest glioma cell killing rate, we attempted to identify the effect factor. IFN- γ was shown to have a capacity to kill glioma cells. IFN- γ concentration was analyzed using ELISA. The GSC group showed a higher concentration of IFN- γ than the IL-13Ra2 group and the non-pulsed group ($P < 0.05$). E/T of 20:1 showed the highest concentration of IFN- γ (Table 6).

Discussion

Previous studies, including ours, have shown that DC vaccines loaded with frozen glioma antigen could be used as a potential therapy for glioma patients. However, its specificity needs to be improved further. In the present study, we have shown that IL-13Ra2 is predominantly expressed in glioma tissue but not in normal brain tissue and its expression is positively correlated with tumor grades. IL-13R is a complex consisting of IL-4Ra, IL-2Rar, IL-13Ra1, and IL-13Ra2, and it has been found that IL-13Ra2 is expressed in several malignant tumors including Kaposi sarcoma and renal carcinoma; however, it is not expressed in normal tissues, thereby indicating its role in tumorigenesis. Kawakami showed that IL-13Ra2 could

Table 4 Killing rate of dendritic cell (DC) groups pulsed with different antigens on glioma cells (%)

E/T	IL-13Ra2 pulsed DCs	GSCs pulsed DCs	Glioma cells antigen	Pure DCs
5:1	16.31 ± 0.92	24.43 ± 0.31	15.6 ± 1.04	5.43 ± 0.51
10:1	32.59 ± 5.43	43.51 ± 5.38	29.21 ± 3.29	7.26 ± 0.41
20:1	42.37 ± 5.18	62.49 ± 6.06	41.28 ± 6.13	8.14 ± 0.46

Table 5 Killing rate of dendritic cell (DC) groups pulsed with different antigens on glioma stem cells (GSCs) (%)

E/T	IL-13Ra2 pulsed DCs	GSCs pulsed DCs	Pure DCs
5:1	6.87 ± 0.71	19.80 ± 2.71	6.42 ± 0.50
10:1	9.21 ± 1.05	25.5 ± 2.24	7.63 ± 0.51
20:1	11.48 ± 0.94	31.46 ± 3.50	10.06 ± 1.21

Table 6 Concentration of IFN- γ in supernatant in dendritic cell (DC) groups pulsed with different antigens, detected by cellular toxicity assay (pg/mL)

E/T	Pure DCs	IL-13Ra2 pulsed DCs	GSCs pulsed DCs
5:1	1012 ± 109	1230 ± 254	1220 ± 137
10:1	1211 ± 225	1444 ± 201	1601 ± 201
20:1	1462 ± 236	1734 ± 337	1856 ± 352

inhibit the JAK-STAT6 signaling pathway by binding to IL-4Ra to promote tumor growth [14]. Joshi reported that about 82% of GBM patients expressed IL-13Ra2; however, this is restricted to the mRNA level [15]. In addition, other groups demonstrated that IL-13Ra2 is mainly expressed in malignant tumors, but not benign ones [16]. These findings, along with our data, indicate that IL-13Ra2 could be a potential specific antigen for DC vaccine therapy for gliomas.

Immunotherapy is one of the promising methods in the treatment of malignant tumors. However, most of the immunotherapies for gliomas are still under clinical trials. Some of the patients showed a good response to immunotherapy involving DC vaccines; however, no benefit was observed in other patients. There are a few probable explanations for this difference: (1) CNS is a relative immune-surveillance organ that lacks immune cells. (2) Glioma presents an immunosuppressive environment, wherein immune cells including DCs and T cells are inactivated by glioma-released factors such as IL-10 and TGF-β [17]. (3) So far, there is no specific antigen that could be used for DC vaccines [18]. DCs are the most powerful antigen presenting cells; however, very few DCs could be found in the peripheral blood and in the brain. GM-CSF and IL-4 have been used to stimulate monocytes to differentiate into DCs, which are capable of producing cytokines. In the current study, we found that IL-13Ra2-pulsed DCs could significantly induce glioma cell death. However, this effect is not valid in case of GSCs. This result may explain why the DC vaccine failed with some patients, who showed a high percentage of GSCs.

The GSC hypothesis indicates that a subpopulation of cells within gliomas has true clonogenic and tumorigenic potential [19]. These cells are not only responsible for tumor recurrence but also resistant to chemotherapy and radiotherapy. GSCs could also influence the tumor microenvironment to impact immunotherapy [20]. Our data showed that IL-13Ra2-pulsed DCs could induce normal glioma cell death but not GSC death, demonstrating that GSCs are resistant to immunotherapy. Interestingly, GSCs can also be treated as the antigen for stimulating DCs. The data obtained in this study showed that GSC-pulsed DCs have a stronger effect on inducing glioma cell death than IL-13Ra2-pulsed DCs and normal glioma cells pulsed-DCs. However, the mechanism underlying this observation is not very clear.

We further analyzed the IFN-γ concentration in DCs after different stimulations, since IFN-γ is believed to be the key factor that induces glioma cell death [21]. It was found that GSC-pulsed DCs released the highest level of IFN-γ. Collectively, we found that both GSCs and IL-13Ra2 could induce glioma cells death probably by releasing IFN-γ, indicating that IL-13Ra2 could be used as a potential antigen for DC vaccines.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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