Abstract. The epidermal growth factor receptor (EGFR) is an attractive target for the immunotherapy of EGFR+ tumors. Adjuvant immunotherapy with cytokine-induced killer (CIK) cells may improve progression-free survival rates in patients suffering from cancer. In the present study, we examined the bispecific antibody anti-CD3 x anti-EGFR (EGFRBi-Ab) for its ability to redirect CIK cells to target EGFR-positive glioblastoma. The specific cytolytic activity of CIK cells armed with EGFRBi-Ab against U87MG-luc cells was evaluated by bioluminescent signal generated using luciferase reporter assay which did not alter the surface molecule expression or proliferation ability of U87MG cells. In contrast to unarmed CIK cells, increased cytotoxic activity of EGFRBi-armed CIK cells against the U87MG-luc target was observed at effector/target (E/T) ratios of 5:1, 10:1, and 20:1. Moreover, EGFRBi-armed CIK cells secreted significantly higher levels of IFN-γ, TNF-α, and IL-2 than their unarmed CIK counterpart cells. Furthermore, in glioblastoma xenograft mice, infusion of the EGFRBi-armed CIK cells successfully inhibited the growth of glioblastoma tumors. The in vitro and in vivo antitumor effects of EGFRBi-armed CIK cells support their clinical use for treatment of glioblastoma in the future.

Introduction

Glioblastoma (GBM) is the most common primary malignancy in brain. The Central Brain Tumor Registry of the United States reports the annual incidence of GBM at ~3.19/100,000 people with a median survival of 15 months (1). The commonly used strategies for treatment include surgery, radiation, and chemotherapy. Although chemotherapy modestly increases survival in patients when surgery and radiotherapy are unsuccessful, the prognosis of GBM is primarily poor (2). Cancer immunotherapy recognized as the fourth antitumor modality has undergone a period of growth following encouraging data regarding its clinical efficacy (3). Immunotherapy approaches are under investigation for GBM, which target the following aspects: enhancing immune response to tumors, inhibiting or destroying molecular or cellular immunosuppressive mediators induced by GBM cells, generating monoclonal antibodies targeting the special tumor antigen in order to eliminate tumor cells, and in vitro expanding tumor-specific lymphocytes naturally arising or manually modified (4). The current overall therapeutic outcomes of GBM are encouraging.

GBM is frequently associated with epidermal growth factor receptor (EGFR) overexpression, and EGFR signaling is important in various types of cancer, including GBM (5). Although noteworthy results of antibody-based therapy in the treatment of melanoma, renal cell carcinoma, and hematologic cancers have been observed, the treatment has not directly benefitted GBM patients (6-8). The antibody specific for EGFR, Erbitux®, showed little effect on GBM patients (9). In early-stage clinical trials, studies have shown promising results for the use of bispecific antibody targeting CD3 and glioma antigen (10-13). Of note, CD8+ T-cell infiltrate is associated with prolonged survival of newly diagnosed GBM patients (14). Additionally, almost half of the T cells
infiltrating GBM specimens were CD56+ T cells (15), and anti-CD3 x anti-GD2 bispecific antibody was able to redirect T-cell cytolytic activity to a neuroblastoma target (16). The above-mentioned observations suggest that adoptive immunotherapy for GBM mediated by bispecific antibodies redirected effector lymphocyte is a promising treatment.

To assess the efficiency of the antitumor effect of the bispecific antibody, a reliable sensitive mouse model is needed. The bioluminescence imaging system as a novel image measuring technology, has been used widely due to its high sensitivity and accuracy in the detection of tumor growth (17). In the present study, we constructed a U87MG-luc cell line that expressed luciferase stably, and a linear correlation was identified between bioluminescent signal intensity and the number of U87MG-luc cells in vitro and in vivo. Clinically approved anti-CD3 antibody was then chemically conjugated with Erbitux®. Considering adjuvant immunotherapy with cytokine-induced killer (CIK) cells can improve progression-free survival rates (18), combination treatments may further improve the survival rates. Thus, the anti-CD3 x anti-EGFR bispecific antibody (EGFRBi-Ab) was used to direct CIK cells to kill the GBM target. Redirected with EGFRBi-Ab, CIK cells exhibited enhanced specific cytotoxicity and cytokine production ability. The efficacy of EGFRBi-Ab-armed CIK cells for the inhibition of EGFR-positive GBM tumor was also investigated in a SCID-Beige mouse model.

Materials and methods

Cell lines and vector construction. Human U87MG glioblastoma were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Colo205-luc, HT-29-luc, A549-luc, NCI-H460-luc, BXPC-3-luc, MDA-MB-231-luc, HeLa-luc and PC-3M-luc cell lines were purchased from Caliper Life Sciences (Hopkinton, MA, USA). Agents for cell culture were from Gibco-Life Technologies (Carlsbad, CA, USA). HeLa-luc and PC-3M-luc cell lines were purchased from Caliper Life Sciences (Hopkinton, MA, USA). Agents for cell culture were from Gibco-Life Technologies (Carlsbad, CA, USA).

The 3.7ln-luc2 plasmid (previously constructed by our laboratory) contained a BirA substrate peptide and c-myc tag linked to a truncated membrane-anchored human low affinity nerve growth factor receptor (ΔLNGFR). The 3.7BirA plasmid (also previously constructed by our laboratory) contained an ER retention signal and BirA enzyme as a reported gene. BirA substrate peptide transported to the cell surface was biotinylated by BirA enzyme to label the target cells (19).

Generation of the U87MG-luc reporter cell line. The 3.7lnLuc2 and 3.7BirA lentiviral transfer vectors are self-inactivating lentivectors used for the separation of the target luciferase reported cells. VSV-G pseudotyped lentiviral vectors stocks were prepared by Lipofectamine 2000 transfection reagent-based transfection of 293T cells with 6 µg of packaging plasmids pLPI, pLP2 and pLp/VSVG (Invitrogen, Carlsbad, CA, USA), respectively, and 3 µg of transfer vector, respectively. The supernatant collected was filtered through a 0.45-µm filter and stored at -80°C. U87MG (1x10^6) cells seeded in a 6-well tissue culture plate 1 day in advance were infected with the viruses at a MOI of 1 by spin inoculation in a centrifuge at 1,800 g for 90 min at 32°C with 8 µg/ml polybrene. Magnetic beads-based cell separation was followed 72 h after post-infection according to the manufacturer's instructions (19). After infection and separation, target luciferase-labeled cells were cultured in a black 96-well plate by limiting dilution. The transduction efficiency and expressed protein tag were monitored by flow cytometry. The bioluminescent imaging signals of selected single-clone U87MG-luc reporter cell lines were measured using the IVIS lumina system (Caliper Life Sciences) in a 96-well plate in culture medium with D-luciferin substrate at a final concentration of 0.15 mg/ml (Bc219-05; Synchem Chemie, Kassel, Germany).

In vitro cell proliferation. The U87MG and U87MG-luc cells were seeded in a 96-well plate and incubated at 37°C overnight. One hundred microliters of fresh medium containing 10 µl Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated for an additional 1 h. The absorbency of U87MG and U87MG-luc cells was measured at 450 nm by a 96-well plate reader (DG5032; Huadong, Nanjing, China) after incubation. According to the manufacturer's instructions, the proliferation of U87MG and U87MG-luc cells was assessed by the absorbance values.

Flow cytometric analysis. Anti-CD3-FITC, anti-CD56-APC, anti-human B7-H1-PE and anti-mouse IgG2a-FITC secondary antibodies were purchased from eBioscience (San Diego, CA, USA). Anti-human B7-H3-PE was purchased from R&D System (Minneapolis, MN, USA), anti-human GD2 was purchased from Millipore (Billerica, MA, USA) and anti-human IgG Fc-PE secondary antibody was purchased from BioLegend (San Diego, CA, USA). The cells were assayed with a Guava flow cytometer EasyCyte (Guava Technologies, Hayward, CA, USA) and the data analysis was carried out with the FlowJo software version 7.6.1 (Tree Star Inc., Ashland, OR, USA).

Preparation of CIK cells from peripheral blood lymphocytes (PBMCs). Peripheral mononuclear blood cells (PBMCs) were separated by Ficoll density gradient centrifugation. Blood was obtained from healthy donors as supplied by the Beijing Blood Bank. CIK cells were expanded 15 days from PBMCs as previously described (20). Briefly, the CIK cells were stimulated by combination of IFN-γ, interleukin-2, interleukin-1α, interleukin-2 (Peprotech, Rocky Hill, NJ, USA) and anti-CD3 mAb (OKT3; eBioscience). Fresh medium containing fresh interleukin-2 was added every 2 or 3 days and the cells were cultured for 15 days prior to being cryopreserved. The study was performed according to the protocols approved by the Biomedical Research Ethics Committee of CAS Key Laboratory of Pathogenic Microbiology and Immunology.

Synthesis of anti-CD3 x anti-EGFR bispecific antibody (EGFRBi-Ab) andarming of CIK cells. Anti-EGFR (Erbitux®; Merek Serono, Darmstadt, Germany) or anti-HER2 (Herceptin®; Roche, Indianapolis, IN, USA) was reacted with sulfo-SMCC and anti-CD3 (OKT3) was reacted with Trast's reagents as previously described (21,22). Cryopreserved CIK cells were thawed, and armed with EGFRBi-Ab at a concentration of 50 ng/10^6 cells at room temperature for 30 min followed by washing the cells to eliminate unbound antibodies. The combination of OKT3 (50 ng/10^6 cells) and Erbitux® (50 ng/10^6 cells) pre-incubated CIK cells were used as unarmed control CIK cells.
In vitro cytotoxicity assay. Cytotoxicity was measured with a luciferase quantitative assay (21-23). Target cells were seeded in triplicate in 96-well microplates at 1x10^4/well prior to the addition of EGFRBi-armed, Her2Bi-armed, or unarmed CIK cells at various effector-to-target (E/T) ratios. Effector and tumor cells were allowed to interact at 37˚C for 18 h. A final concentration of 0.15 mg/ml D-luciferin was added to each well. The IVIS lumina system was used to measure the bioluminescent image signal.

ELISA assay. U87MG-luc cells were seeded (1x10^4/well) in 96-well microplates in triplicate overnight. The medium was removed, and fresh medium or medium containing EGFRBi-armed CIK or control CIK cells was added to wells at an E/T of 20:1. The CIK and target U87MG-luc cells were incubated at 37˚C for 18 h. The supernatants were collected and production of IFN-γ, TNF-α, and IL-2 was quantified by the human cytokine ELISA kit (eBioscience) according to the manufacturer's instructions.

In vivo antitumor effect of EGFR-BiAb-armed CIK cells. Female eight-week-old SCID-Beige mice were purchased from the Peking University Health Science Center (Beijing, China). The U87MG-luc cells (5x10^6) were injected subcutaneously into the dorsal region of female mice (6 weeks old, n=5) and the bioluminescence signal was monitored by the in vivo imaging system at the indicated days after inoculation.

Figure 1. Construction of plasmid and establishment of U87MG-luc cells. (A) Diagrammatic structure of two lentiviral vectors: 3.7lnluc2 and 3.7BirA. (B) U87MG cells infected with 3.7lnluc2 and 3.7BirA lentivirus were isolated by DynaBeads, and positive cells were harvested. (C) The bioluminescence image signal was measured with the IVIS lumina system. Serial dilution of U87MG-luc cells was plated in a black 96-well plate in PBS containing D-luciferin substrate at a final concentration of 0.15 mg/ml. Pearson’s correlation coefficient (R) was used to analyze between the luciferase quantity and living cell number is 0.99. (D) FACS analysis of c-Myc expression on selected U87MG-luc cells. (E) U87MG-luc cells (5x10^6) were injected subcutaneously into the dorsal region of female mice (6 weeks old, n=5) and the bioluminescence signal was monitored by the in vivo imaging system at the indicated days after inoculation. (F) Mean tumor luminescence signal of inoculated SCID-Beige mice was measured by photons per second.

Statistical analysis and reproducibility. Experiments were repeated at least three times. Data were analyzed using Graphpad Prism 5 software and were presented as the means ± SDs. Unpaired Student’s t-test (two-tailed) or the Mann-Whitney test was used for comparison of two groups where appropriate. One-way analysis of variance (ANOVA) followed by Dunnett’s post hoc were used for multiple comparisons. Pearson’s correlation coefficient (R) was analyzed. P<0.05 was considered statistically significant. The number with a significant difference from a control is denoted by an asterisk in the figures.
Results

Establishment of human U87MG-luc glioblastoma cell line stably expressing luciferase. To establish U87MG-luc cells, 3.7ln-luc2 and 3.7BirA lentivirus plasmids (Fig. 1A) were constructed and used to produce pseudotyped lentiviruses. The U87MG cells infected with the lentivirus were able to express luciferase protein and process the transmembrane protein on the surface of cells. After adding the substrate d-biotin, BirA-tag was biotinylated, attached to streptavidin beads, and positive cells were subsequently separated (Fig. 1B). A single U87MG-luc clone was obtained by serial limiting dilutions in 96-well plates and the luciferase expression was detected and analyzed by the IVIS lumina imaging system with Living Image software. We seeded a number of U87MG-luc cells in 96-well plates ranging from 1,562 to 100,000 (1,562, 3,125, 6,250, 12,500, 25,000, 50,000 and 100,000) per well. The results showed that there was a good correlation between the level of luciferase activity and the number of U87MG-luc cells (Fig. 1C). The linear correlation between the luciferase activity-related bioluminescent signal and the number of cells is shown in Fig. 1C ($R^2=0.99$). Moreover, the expression of myc-BirA-tag was detected by FACS staining with anti-myc antibody (Fig. 1D).

To investigate whether U87MG-luc cells could be used to establish a subcutaneous tumor model in SCID-Beige mice, 5x10^6 U87MG-luc cells were injected subcutaneously in the dorsal thigh of female mice (n=6), and the bioluminescent signal reflecting tumor growth was monitored and analyzed using the IVIS lumina imaging system with Living Image software at the indicated days (Fig. 1E). Mean tumor luminescence was also calculated (Fig. 1F). Tumor size was measured and a linear correlation between mean luminescence and mean tumor size was observed ($R^2=0.99$, data not shown).

Luciferase gene has no effect on cell proliferation and surface molecular expression of U87MG cells. To investigate whether the luciferase gene itself had a negative effect on U87MG cell growth in vitro, proliferation assays were performed (Fig. 2A). To measure the proliferation of cells stably expressing luciferase, the proliferation rates of U87MG and U87MG-luc cells were detected by a CCK-8 assay as described in Materials and methods. The results showed that there was no significant difference in proliferation between the U87MG and U87MG-luc cells.

We detected the expression of EGFR, Her2, B7-H3, B7-H1, and GD2, the attractive molecules used as the targets for glioblastoma, with the specific antibody on the surface of U87MG and U87MG-luc, respectively. As shown in Fig. 2B, a high expression of EGFR, B7-H3 and GD2 and a low expression of B7-H1 was detected on U87MG and U87MG-luc cells. By contrast, Her2 was not detected on the cells. Moreover, the
The expression level of these molecules was similar on the surface of U87MG and U87MG-luc cells. Taken together, these results indicated that the luciferase gene did not affect the proliferation or target the molecular expression of U87MG cells.

Therefore in the subsequent experiment, we selected one of the molecules with a high expression on U87MG-luc and EGFR, as the target for arming CIK cells.

Preparation and characterization of EGFRBi-Ab and CIK cells. Bispecific antibody EGFRBi-Ab was prepared as described in Materials and methods (Fig. 3A). Firstly, the binding specificity of EGFRBi against EGFR was assessed. U87MG-luc cells were stained with EGFRBi-Ab or a combination of OKT3 and Erbitux® (used as the unarmed control for EGFRBi-Ab). Anti-mouse IgG2a-FITC was then added to detect the CD3 moiety of EGFRBi-Ab. Positively stained cells were detected in 88% of the U87MG-luc population with a mean fluorescent intensity (MFI) of 24.

To produce a sufficient number of effector cells, PBMCs from buffy coat were stimulated by the combination of IFN-γ, IL-1α, IL-2 with OKT3 for 15 days as described in Methods and materials. The CIK cells were then quantitatively analyzed by FACS. As shown in Fig. 3D-F, the CIK cells contained almost 96.74±1.49% of CD3$^+$ cells, i.e., 20.02±9.4% of CD3$^+$ CD56$^+$ cells (Fig. 3D), 36.1±8.37% of CD3$^+$ CD4$^+$ (Fig. 3E), and 61.46±7.43% of CD3$^+$ CD8$^+$ (Fig. 3F) approximately. For the CD3$^-$ population, the majority of cells were CD56$^-$, 2.73±1.43% (Fig. 3D). Overall, these data suggested that the CIK cells mainly comprised T cells and NK T cells with a small population of NK cells.

Cytotoxicity effects of EGFRBi-armed CIK cells with IFN-γ TNF-α and IL-2 production on glioblastoma cells. The amount of EGFRBi-Ab required to arm CIK cells ranged from 5 to 500 ng/10$^6$ cells, with 50 ng and 500 ng/10$^6$ cells showing a similar cytotoxicity. Therefore, we selected 50 ng/10$^6$ cells as the concentration of EGFRBi-Ab for all the subsequent experiments, and CIK cells mixed with individual OKT3 and Erbitux® were considered unarmed CIK controls. Cytotoxicity assays were performed at E/T ratios of 5:1, 10:1 and 20:1 for
18 h. The bioluminescent images correlated with the number of living U87MG-luc cells (Fig. 4A). After 18-h incubation with EGFRBi-armed CIK or unarmed CIK or Her2Bi-armed CIK cells, bioluminescent image signal expressed in photons per second was converted into a living cell number and the cytotoxicity assays were calculated at the indicated E/T ratios. As shown in Fig. 4B, an increasing E/T ratio was correlated directly with the percentage of cytotoxicity in all the tested CIK effectors. The percentage of cytotoxicity of EGFRBi-armed CIK cells was significantly greater than that of the other groups at each E/T ratio.

To analyze the cytokines along with the cytotoxicity, supernatants of cell cultures were analyzed for cytokine production at an E/T of 20:1. As shown in Fig. 4C-E, significant increase was observed for IFN-γ, TNF-α and IL-2 secretion in EGFRBi-armed CIK cells over their unarmed CIK counterparts or Her2Bi-armed CIK cells when co-cultured with U87MG-luc cells.

Cytotoxicity effects of EGFRBi-armed CIK cells on different tumor cell lines. We assessed the ability of EGFRBi-armed CIK cells to respond to a wide range of human EGFR-positive carcinoma, including colorectal (Colo205-luc and HT-29-luc), pancreatic (BXPC3-luc), lung (A549-luc and NCI-H460-luc), breast (MDA-MB231-luc), cervical (HeLa-luc), and prostate (PC-3M-luc) cancer. After 18-h incubation with EGFRBi-armed CIK or unarmed CIK cells (Fig. 5), the percentage of cytotoxicity with EGFRBi-armed CIK cells was significantly greater than that with unarmed control effectors at E/T ratios of 5:1 and 10:1 in the EGFR-positive cancer cells.

EGFRBi-armed CIK cells inhibit U87MG tumor growth in SCID-Beige mice. To determine whether EGFRBi-armed CIK cells suppressed tumor growth in vivo, SCID-Beige mice were engrafted subcutaneously with U87MG-luc cells. On the following day, the mice were treated with unarmed control CIK cells or EGFRBi-CIK cells, respectively. After injection, the mice were given no further treatment but were monitored with bioluminescent imaging on the indicated day. This bioluminescent imaging model allows the monitoring of tumor cell fate as early as the first few days after inoculation, when tumor formation cannot be detected by palpation, and three representative mice of each group were shown (Fig. 6A). The mice treated with unarmed CIK cells showed stronger lumi-
nescence than the EGFRBi-armed CIK cells. Furthermore, after comparing the mean luminescence of the two groups, significant differences in inhibition of tumor growth were observed between them (Fig. 6B). Therefore, compared with the unarmed control CIK cells, EGFRBi-armed CIK cells inhibited tumor growth in vivo.
Discussion

Although the therapeutic antibody for EGFR, such as Erbitux®, significantly improved survival rates in patients with metastatic colorectal cancer, these results could not be duplicated in GBM (9). Intradatum heterogeneity and EGFR pathway redundancy limited the clinical utility of the antibody-based therapy in GBM (24). Bispecific antibody comprises an immune effector cell-specific antibody and its hetero-conjugated mAb specific to a selected tumor-associated antigen (TAA). Such a bispecific antibody may redirect immune- potent effector cells to target tumor cells. EGFR is an ideal candidate used as a target in various types of tumor imaging and antibody-based therapeutic approaches. Preclinical findings have shown that arming activated T cells with bispecific EGFR antibody can target EGFR+ cancers (25). In addition, adjuvant immunotherapy with CIK cells may prevent recurrence, and improve progression-free survival rates, and the quality of life (18). The combination of CIK cell therapy with conventional adjuvant or palliative therapies was superior to the standard therapy alone, indicating the benefit of CIK cell therapy for cancer patients (26). In our study, the CIK cells comprised T cells and NK T cells, as well as a small population of NK cells.

In the present study, we armed CIK cells with bispecific Ab and tested whether EGFR is a useful target for GBM. The results showed that, EGFRBi-armed CIK cells exhibited significant cytotoxic activity against human GBM U87MG cells in vitro. We also validated the specific lysis of a wide range of EGFR-positive human tumor cells, including lung, colorectal, pancreatic, breast, cervical, and prostate cancer by EGFRBi-Ab redirected CIK cells in vitro. Additionally, EGFRBi-armed CIK cells secreted a higher level of IFN-γ, TNF-α, and IL-2 than unarmed CIK cells. It is conceivable that arming leads to binding specifically to tumor cells and the triggering of CIK cell activation and cytokine secretion. The increase in tumoricidal cytokines suggested that armed CIK cell infusions may vaccinate patients against their own tumors. Infusion of EGFRBi-armed CIK cells also markedly inhibited the growth of GBM cells in the xenograft mouse model.

The in vivo bioluminescence imaging (BLI) system has developed rapidly in recent years. With the sensitive, non-invasive, and quantitative system of BLI technology, it is possible to localize and monitor the orthotopic and metastatic growth of tumor in vivo (27). Specifically, BLI is widely used in cancer research and therapy (28). In the present study, we initially constructed a stable human U87MG-luc GBM cell line that expressed a high level of luciferase. As our data has shown, there was a good correlation between the luciferase activity and the number of cells. Furthermore, similar to the study by Tiffen et al (29), the luciferase gene did not affect the surface expression of EGFR, Her2, B7-H3, B7-H1, GD2, and the proliferation of GBM U87MG cells. We also established a xenograft subcutaneous human GBM tumor model in SCID-Beige mice with U87MG-luc cells and supplied a sensitive model for investigation of the pathogenesis of GBM, and assessment of the efficiency of immunotherapies.

In addition to adoptive cell therapy, the immune checkpoint blockade in passive immunotherapy has been successful in the treatment of various types of cancer, thereby encouraging a resurgence of interest in GBM (30). A new approach being evaluated in clinical trials involves the use of monoclonal antibody to block immunosuppressive molecules such as PD-1 expressed by T cells. The monoclonal antibody specific for PD-1 is a promising treatment for GBM due to its tumor-expressed ligand PD-L1 (B7-H1), for predicting the efficacy of targeting the PD-1/PD-L1 pathway (31). In our study, administration of EGFRBi-armed CIK cells suppressed established tumor growth in vivo, although this did not completely eradicate the tumor cells. This occurred due to the insufficient persistence of armed-CIK cells. Function sustaining and trafficking of human CIK cells in the xenograft tumor model was more difficult than that of CIK cells in the immune system of the patient.

In summary, to the best of our knowledge, this study has shown for the first time that EGFRBi-Ab is capable of enhancing CIK cells ability to kill GBM and other EGFR-positive cancers. In addition, a sensitive model for evaluating antitumor effect for GBM has been generated. The in vitro and in vivo antitumor effect of EGFRBi-armed CIK cells supports their further clinical use for the treatment of GBM.

Acknowledgements

This present study is funded by the grants from the Ministry of Science and Technology of China (S&T major program no. 2012ZX1004701-001-002), the Basic Research Program of China (973, no. 2013CB531502) and the National Nature Science Foundation of China (no. 31400754, 31370889, 81273270, 81041110, 81471590 and 81402549).

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