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Enhancing therapy of B16F10 melanoma efficacy through tumor vaccine expressing GPI-anchored IL-21 and secreting GM-CSF in mouse model

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ABSTRACT

In the present study, we developed the tumor vaccine expressing IL-21 in the GPI-anchored form together with secreting GM-CSFs and investigated its antitumor efficacy in C57BL/6 mouse model. The fusion genes containing IL-21 and the GPI anchor signal sequence were acquired by overlaping PCR, inserted into the downstream of two multi-clone sites in recombinant plasmid pRSC/GM-CSFs to form pRSC/IL-21-gpi-GM-CSFs that was transfected into the B16F10 cells. The tumor cell vaccine B16F10/IL-21-gpi-GM-CSFs was identified by reverse transcription PCR, IFA and FCM, respectively. The results showed that the pRSC/IL-21-gpi-GM-CSFs had no cell cycle and proliferative state impact on the B16F10 cells after transfected, and that the tumor vaccine B16F10/IL-21-gpi-GM-CSFs increased the cytotoxicities of NK cells and $CD8^+$CTL, enhanced the level of serum IFN-γ, augmented therapy of tumor effect and prolonged survival time in the tumor-bearing mice immunized with the tumor vaccine B16F10/IL-21-gpi-GM-CSFs. The data that we presented here provided a rationale and practical platform for clinical testing of enhancing cell therapy of B16F10 melanoma efficacy by modified tumor vaccine expressing GPI-anchored IL-21 and secreting GM-CSF.

1. Introduction

Although new immunotherapeutic options for all kinds of cancers have been developed within recent years, vaccination is still a promising emerging treatment option. The use of tumor cells genetically engineered to express immune stimulatory cytokines is a suitable approach to induce antitumor immune responses by a paracrine effect [1–3]. The autologous cytokine-producing tumor vaccine is one common useful tumor vaccine. Among various tumor vaccine approaches, IL-21 is involved in T and NK cell activation and effector response in the tumor vaccine approaches [2]. IL-21 is widely applied to significantly augment antitumor immunity in multiple murine tumor models and clinical trials, such as metastatic lymphoma [4], melanoma [2,5], thymoma [6], renal cell carcinoma [7], neuroblastoma [8]. Analogously, GM-CSF has shown promising results as a cytokine adjuvant for antiviral vaccines and in various models of tumor gene therapy [9]. GM-CSF exerts its effect on innate and acquired immunity with the most prominent action in NK cells and $CD8^+$ memory T cells. Therefore, many authors have proposed that GM-CSF could be a good candidate for augmenting the efficacy of vaccination strategy [9].

Recently, a growing body of literature shows that the administration of two kinds of cytokines as an adjuvant elicit cellular and humoral responses markedly in antitumor immunity [10,11]. The ability of these cytokines to regulate antitumor immunity in mice has generated considerable interest in understanding their mode of action. GPI membrane anchors of cell-surface glycoproteins have been attached to confer functional properties that are different from their transmembrane-anchored counterparts. A wide range of cell-surface proteins including enzymes, encoding proteins, surface antigens, cytokines, and adhesion molecules are attached to the plasma membrane via GPI anchors [12,13]. GPI-anchored membrane cytokines have been shown to play an important role in host immune responses against tumor cells. To optimize the therapeutic efficacy and lower the side effects of IL-21, we have investigated the immunotherapy of tumor efficacy through tumor cell vaccine that was combined IL-21 in the GPI-anchored form with secreting

Abbreviations: GPI, glycosylphosphatidylinositol; IL-21, interleukin-21; GM-CSF, granulocyte macrophage colony stimulating factor; CTL, cytotoxic T lymphocytes; NK, nature killer cells; IFA, immunofluorescence assay; FCM, flow cytometry; RT-PCR, reverse transcription polymerase chain reaction; SPF, S-phase fraction; PI, proliferation index; MACS, magnetic activated cell sorting.

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GM-CSF (GM-CSFs) in a highly malignant and poorly immunogenic murine melanoma model. For the purpose, we firstly developed a membrane-anchored IL-21 by the GPI and expressed IL-21 on tumor membrane vesicles to deliver and confine IL-21 to the vaccination site in C57BL/6 mouse model and then prepared the tumor vaccine expressing IL-21-GPI-anchored and GM-CSFs simultaneously. The experiment results showed that the tumor cell vaccine B16F10/IL-21-gpi-GM-CSFs had an effective of antitumor immune therapy in the tumor-bearing mice. The goal of our study was to design a promising new strategy for combining tumor cell vaccine with other therapeutic approaches, as well as novel perspectives in the treatment of the patients with melanoma in future.

2. Materials and methods

2.1. Primers and plasmids

The sequence of PCR sense primer 1 for GPI gene is 5′-ATCAGCATCTCTCCACCGACGCC GCGCA-3′, which is complementary to anti-sense primer 4; the anti-sense primer 2 for GPI gene is 5′-ATGC***AAGCTT***TCAGG GAGCAGTG GCCGT-3′ cDNA oligos with Hind III site. The sequence of PCR sense primer 3 for IL-21 gene is 5′-GGC***GCTAGC***ATGGATCGCCTCC TGATTAGACTT-3′ cDNA oligos with Nhe I site, which is easily inserted into plasmid pcDNA3.1 [14]. Anti-sense primer 4 for IL-21 gene is 5′-TGCGCGGCGTCGGTGGAGAGATG CTGAT-3′. The recombinant plasmid pcDNA3.1 containing mIL-21 gene and blank pcDNA3.1 are respectively stored in our lab [15]. The sequence of PCR sense primer 5 for IL-21-gpi gene is 5′-GTAA***GCGGCCG***CATGGATCGCCTCCTGATTAGACTTCGT-3′ cDNA oligos with Not I site and the anti-sense primer 6 is 5′-GCAC***CTCGAG***TCAGGGAGCAGTGGCCG-3′ cDNA oligos with Xho I site, which is easily inserted into the downstream of two multi-clone sites in recombinant plasmid pRSC/GM-CSFs for constructing pRSC/IL-21-gpi-GM-CSFs [16].

2.2. Animal and cell lines

The B16F10 murine melanoma cell line is syngeneic in C57Bl/6 mice and was a generous gift from professor Pingsheng Chen, Department of Pathology, School of Basic Medical Science, Southeast University, Nanjing, China. YAC-1 cell line is moloney leukemia-induced T-cell lymphoma of A/Sn mouse origin and was purchased from the Cellular Institute in Shanghai, China. These cells are cultured at 37 °C in 5% CO_2 in complete media consisting of RPMI 1640, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FCS). C57BL/6 mice of 5–6 weeks of age were acquired from the University of Yangzhou of China. All the experiments were performed in compliance with the guidelines of the Animal Research Ethics Board of Southeast University, China.

2.3. Constructions and transfections

The construction of recombinant pRSC/1-IL-21-gpi and transfections of the B16F10 cell lines were according to our previous report [14]. The constructions of pRSC/IL-21-gpi and pRSC/IL-21-gpi-GM-CSFs were briefly programmed as following process. The gene GM-CSFs was amplified from the recombinant pRSC/GM-CSFs by PCR and then was inserted into the multi-clone sites A in an eukaryotic plasmid pRSC with two multi-clone sites. The IL-21-gpi gene was amplified from pCDNA3.1/IL-21-gpi by PCR and then was inserted into the multi-clone sites B in the recombinant plasmid pRSC as shown in Fig. 1. The different recombinants or blank plasmid as control was separately transfected into the B16F10 cells using Lipofectamine™ 2000 reagent (Invitrogen, USA) according to the manufacturer's protocol. Stable expressing IL-21gpi cell lines, or

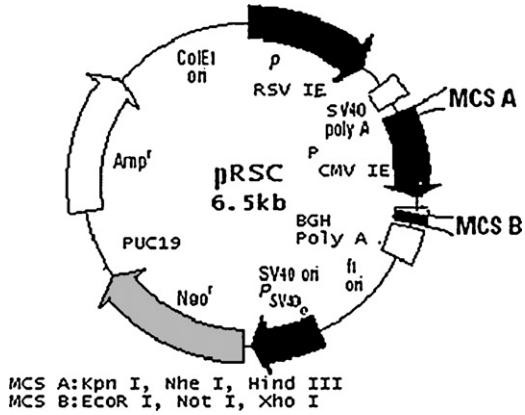

Fig. 1. Draft of constructing pRSC/IL-21-gpi-GM-CSFs. There are two multi-clone sites (MCS) in eukaryotic plasmid pRSC. The gene GM-CSFs was inserted into MCS A. The IL-21-gpi gene was inserted into MCS B.

IL-21-gpi-GM-CSFs cell lines were respectively selected with RPMI containing 800 μg/ml G418 (Clontech, CA). After 10 days, G418-resistant clones were selected, and we named B16F10/IL-21-gpi cells for cells expressing IL-21-gpi (GPI-anchored IL-21), B16F10/IL-21-gpi-GM-CSFs cells for cells expressing IL-21-gpi and GM-CSFs and B16F10 cells transfected with blank plasmid for no expressing any cytokines. The constructed recombinants were respectively identified by endonuclease digestion and DNA sequencing.

2.4. Analysis expressions of IL-21-gpi and GM-CSFs

RT-PCR was programmed using the method from the published report [17]. Briefly, total cellular RNA was extracted from 1×10^6 B16F10 cells transfected with the different recombinants by using Rneasy Mini Kit (Qiagen, CA) according to the manufacture's instructions, and all cellular RNA was put with DNAase for 1 h at room temperature in order to get rid of DNA contamination. cDNA was synthesized with the reverse SuperScript Choice System (Invitrogen, CA). cDNAs of GM-CSFs, cDNAs of IL-21-gpi and cDNAs of B16F10 cells were respectively amplified by PCR with the aforementioned primers. Amplification of the genes of GM-CSFs and IL-21-gpi were identified by 1.5% the agarose gel electrophoresis. In IFA assay, the B16F10/IL-21-gpi cells and the B16F10 cells fixed with 4% paraform for 10 min (min) and atmospheric drying, then rinsed three times in phosphate-buffered saline (PBS). After that, the cells was blocked with Tris buffered saline with Tween containing 5% bovine serum albumin (BSA) at 37 °C for 40 min and then incubated with goat anti-mouse antibody at 4 °C overnight, rinsed three times in PBS, and then the cells was incubated with rat anti-goat antibody labeled with fluorescein isothiocyanate (FITC, eBioscience company, USA) at 37 °C for 1 h. Finally, the B16F10/IL-21-gpi cells or the B16F10 cells on the glass was mounted with containing 50% glyceric PBS, and was then observed by the fluorescence microscope [18]. FCM (BD company, USA) was performed in the FCM Laboratory, at the Center for Clinical Medicine, Southeast University, according to relevant protocols. Thawed B16F10/IL-21-gpi cells, B16F10/GM-CSFs cells, B16F10/GM-CSFs-IL-21-gpi cells and B16F10 cells were respectively suspended in Hanks balanced salt solution (HBSS; Sigma–Aldrich, Poole, United Kingdom) plus 2% BSA at 100 ml. The cells were incubated with 2 μl rat anti-mouse IL-21 monoclonal antibody (final concentration, 0.2 μg/ml) and/or anti-mouse GM-CSF antibody labeled with phycoerythrin (PE) for 30 min at room temperature and then incubated with 3 μl goat anti-rat-FITC stored at room temperature in PBS containing 0.5 μg/ml propidium iodide for 30 min in a volume of 1 ml. The cells were then rinsed twice in PBS, and immediately analyzed by FCM [19,20].