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Combination therapy with dendritic cell vaccine and IL-2 encapsulating polymeric micelles enhances intra-tumoral accumulation of antigen-specific CTLs

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ABSTRACT

Dendritic cell (DC) vaccine is a promising immunotherapy for cancer due to its ability to induce antigen-specific CTLs efficiently. However, a number of clinical studies have implied insufficient therapeutic benefits with the use of MHC class 1 restricted peptide-pulsed DC vaccine. To enhance the clinical efficacy, we examined combination therapy of DC vaccine pulsed with OVA peptide and intravenous low dose unmodified IL-2 (IL-2 solution) administration against EG7 tumor-bearing mice. Unexpectedly, no additional effects of IL-2 solution were observed on CTL induction and the therapeutic effects of DC vaccine, possibly because of the short half-life of IL-2 in plasma. Therefore, we generated IL-2-encapsulating polymeric micelles (IL-2 micelle), which showed prolonged IL-2 retention in the circulation after intravenous administration compared with IL-2 solution. When mice were treated with OVA peptide-pulsed DCs in combination with IL-2 micelle, OVA-specific CTLs were efficiently induced in the spleen in comparison with DC vaccine combined with IL-2 solution or DC vaccine alone. In addition, combination therapy of DC vaccine and IL-2 micelle against EG7 tumor-bearing mice induced the efficient accumulation of antigen-specific CTLs into the tumor and marked anti-tumor effects. Thus, the administration of IL-2 micelle can significantly enhance DC vaccine efficacy against tumors.

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1. Introduction

DCs are potent antigen presenting cells (APCs) that play critical roles in the activation of naïve T cells. DCs can induce tumor antigen-specific CTLs, which kill tumor cells directly, through the presentation of antigen-derived peptides on MHC class I. Therefore, DC-based vaccination is thought to be a promising strategy for cancer immunotherapy. However, many clinical studies of DC-based vaccination suggest insufficient therapeutic benefits [1]. The anti-tumor effects of DC vaccines might be enhanced by combination with other therapies, such as low molecular-weight compounds or cytokines, such as IL-2. The enhancement of therapeutic efficacy on DC vaccine by IL-2 remains to be elucidated despite several clinical studies [2–4]. IL-2 is a well-known multifunctional cytokine that influences various lymphocyte subsets including CTLs, Treg cells and NK cells during differentiation, immune responses and homeostasis [5]. Recombinant IL-2 (Proleukin) is a Food and Drug Administration-approved drug for patients with metastatic melanoma and renal cell carcinoma and has been used in combination with DC vaccine because of its ability to expand T cell numbers. It was reported that anti-tumor effect of DC vaccine was enhanced with a combination of low dose IL-2 in animal model using MCA-207 sarcoma [6]. In this experiment, however, twice daily administrations of IL-2 for several days were required for maximal efficacy [6], possibly because of the short half-life of IL-2 [7].

Recently, various drug delivery systems (DDS) have been developed to improve pharmacokinetics and efficacy and/or to decrease dosing frequency and side effects for many drugs. Polymeric micelles consisting of polyethyleneglycol (PEG)-poly (amino acid derivative) block copolymers, originally developed by Dr. Kataoka's group [8], are a promising DDS. Block copolymers form a miceller structure spontaneously in aqueous medium and can incorporate various kinds of drugs into their inner core with relatively high stability. Previously, we reported improved pharmacokinetics and in vivo efficacy of recombinant human G-CSF encapsulated in polymeric micelles [9].

In the current study, we designed IL-2 encapsulating polymeric micelles (IL-2 micelle) comprised of PEG-polyGlutamate (PEG-pGlu) block copolymer to improve pharmacokinetics of unmodified IL-2 (IL-2 solution) and evaluated efficacy of combination therapy with DC vaccine and IL-2 micelle. Results showed that IL-2 micelle extended plasma retention of IL-2 and induced antigen-specific CTLs efficiently, leading to improved efficacy when combined with DC immunization. Thus, the

Abbreviations: PEG, Polyethylene glycol; AUC, Area under the concentration–time curve; MRT, Mean residence time; EPR effect, Enhanced permeability and retention effect.

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combination of DC vaccine and IL-2 micelle could be a promising novel therapy for cancer patients.

2. Materials and methods

2.1. Animals

7-week-old female C57BL/6 mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). 5–6-week-old male Wister rats were purchased from Charles River (Yokohama, Japan). All animal experiments were performed in accordance with our institutional guidelines.

2.2. Antibodies and regents

IL-2 (Proleukin) was obtained from Novartis (Basel, Switzerland). FITC-conjugated anti-CD4, CD8, CD86 and NK-1.1, PE-conjugated anti-CD3, CD11c and Foxp3 antibodies and mouse Foxp3 buffer set were from BD Biosciences (San Jose, CA). H-2K^b OVA peptide (OVA$_{257–264}$: SIINFEKL), which is MHC class 1 restricted, and H-2K^b OVA tetramer-SIINFEKL were from MBL (Nagoya, Japan). 7-Aminoactinomycin D (7-AAD) was purchased from Life Technology (Carlsbad, CA). Recombinant murine GM-CSF and IL-4 were from PeproTech (Rocky Hill, NJ). Lipopolysaccharide (LPS) was from Sigma Aldrich (Irvine, CA). Collagenase D and DNase I were purchased from Roche (Basel, Switzerland).

2.3. Cell culture and preparation

EG7 tumor cells, which are expressing OVA, were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium containing 10% FBS supplemented with Geneticin (Life Technology).

DCs were prepared from bone marrow (BM) as previously described [10]. Briefly, BM cells from C57BL/6 mice were cultured with medium containing 20 ng/ml of GM-CSF for 9 days and subsequently matured in the medium containing 20 ng/ml of GM-CSF and IL-4 and 1 μg/ml of LPS for additional one day. These harvested cells contained more than 80% of CD11c+ CD86+ cells and used as mature DCs. In preparation of peptide-pulsed DCs, mature DCs were cultured in the presence of H-2K^b OVA peptide at 1 μg/ml for 5 h at 37 °C. After washing with PBS three times, the DCs were used for immunization.

For preparation of cell suspensions from tumor tissues, resected tumor tissues were minced and digested in RPMI 1640 containing 20% of FBS, 1 mg/ml of collagenase D and 25 μg/ml of DNase I for 1 h at 37 °C. After passing through a 70-μm cell strainer (BD Biosciences), the cells were washed three times with RPMI 1640, hemolyzed with 0.83% NH_4Cl, washed with PBS and subjected to OVA tetramer assay. The number of intra-tumoral OVA-specific CTLs was corrected per tumor volume.

2.4. Preparation of IL-2-encapsulating polymeric micelles (IL-2 micelle)

PEG-pGlu block copolymer partially substituted with octyl group at carbonyl carbon of amino acid through ester linkage was synthesized at NanoCarrier Co., Ltd (Kashiwa, Japan). The mean molecular weight of PEG is approximately 10 kDa, and the mean number of Glu residues is approximately 40. Empty polymeric micelles were prepared by dispersion of the polymer in 20 mM sodium citrate buffer (pH 5.0) containing 10% (*w*/*v*) sucrose, followed by sonication and filtration through a Millipore 0.22-μm filter. IL-2 was encapsulated into the micelles at a ratio of 5% (*w*/*w*) to the polymer, as described previously [9]. Micelle diameter was determined by Zeta-Sizer Nano-ZS (Malvern, Worcestershire, UK) at 25 °C. The IL-2 content of the micelles was determined by HPLC. The micelles were stored at −80 °C prior to use.

2.5. Pharmacokinetics of IL-2 micelle

Rats were administered with IL-2 micelle or IL-2 solution intravenously at 3.5×10^5 IU/kg (a dose of IL-2 micelle is hereafter expressed as IL-2 equivalent IU/kg of body weight per injection). The blood was collected at 5 min, 1, 3, 6, 12, 24 and 48 h and the plasma was separated by centrifugation and stored at −80 °C until analysis.

To obtain the released IL-2 concentration, plasma samples were subjected to gel filtration chromatography. IL-2 concentrations in these samples were determined by ELISA. The AUC and the MRT were calculated based on a 1- and non-compartmental model for IL-2-solution and -micelle group, respectively.

2.6. Flow cytometry

Before staining with fluorescent antibodies and/or tetramer, FcγR was blocked with anti-CD16/CD32 antibody. For analysis of NK cells, splenocytes were stained with FITC-anti-NK1.1 and PE-anti-CD3 antibodies. NK cells were defined as NK1.1$^+$CD3$^-$ cells. To identify Treg cells, splenocytes were stained with FITC-anti-CD4 antibody, fixed and permeabilized using mouse Foxp3 buffer set according to the manufacturer's protocol and stained with PE-anti-Foxp3 antibody. Treg cells were identified as CD4$^+$Foxp3$^+$ cells. For MHC class I tetramer assay, splenocytes or cells prepared from tumor tissues were stained with PE-conjugated H-2K^b OVA tetramer and FITC-anti-CD8. To identify live cells, unfixed cells were stained with 7-AAD. The cells were acquired by EPICS XL (Beckman Coulter, Brea, CA).

2.7. In vivo CTL induction

C57BL/6 mice were injected intravenously with OVA peptide-pulsed or unpulsed DCs (3×10^5) at days 0 and 14. From days 2 to 26, vehicle, empty micelle, IL-2 solution (3.5×10^5 IU/kg) or IL-2 micelle (3.5×10^5 IU/kg) were administered intravenously every 2 days except day 14. Splenocytes were prepared and subjected to tetramer assay at days 0, 8, 14, 18, 22, 26 and 28 and analyzed for the frequency of NK cells and Treg cells at day 22.

2.8. Therapeutic experiments

Mice were inoculated with EG7 tumor cells (5×10^5) subcutaneously at day 0. Mice were immunized with DCs (3×10^5) intravenously at day 4 and administered intravenously with vehicle, empty micelle, IL-2 solution at 3.5×10^5 IU/kg (×1) or 10.5×10^5 IU/kg (×3) or IL-2 micelle at 3.5×10^5 IU/kg (×1) at days 6, 8 and 10. Tumor volume was measured with a micrometer caliper two or three times a week. In some experiments, mice were sacrificed at day 12 to analyze intra-tumoral accumulation of CTLs.

2.9. Statistical analysis

Analysis was performed with a Tukey–Kramer method. $p < 0.05$ was considered significant. Q3

3. Results

3.1. Anti-tumor effect of OVA peptide-pulsed DC vaccine in combination with IL-2 solution

To enhance anti-tumor effects of DC vaccine, we first evaluated the combination therapy of OVA peptide-pulsed DC vaccine and IL-2 solution against EG7 tumor-bearing mice. Mice were inoculated with EG7 cells at day 0 and immunized with OVA peptide-pulsed or unpulsed DCs intravenously, 4 days later. Then mice were administered IL-2 solution intravenously three times at days 6, 8 and 10. As reported previously [11], OVA peptide-pulsed, but not unpulsed DC immunization without IL-2

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