Journal of Controlled Release 158 (2012) 286–292

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

Targeted delivery of oligonucleotides into tumor-associated macrophages for cancer immunotherapy

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ARTICLE INFO

Article history:
Received 10 August 2011
Accepted 11 November 2011
Available online 18 November 2011

Keywords:
Tumor associated macrophage
Nucleic acid drugs
Targeting delivery
Cancer immunotherapy

ABSTRACT

Tumor-associated macrophages (TAMs) have been proven to be a driving force in the initiation, proliferation, metastasis and angiogenesis of various tumors. Specifically, alterations in the functions of TAMs exhibited inhibitory effects on tumor growth. However, there is currently no research being conducted on the targeting delivery of drugs into TAMs for cell-specific tumor immunotherapy. In the present study, we developed a TAMs targeted delivery system that is triggered by the acidic microenvironment in the tumor to release a TAMs-recognizing nano-complex loaded with oligonucleotides. By using this system, we demonstrated a significant anti-tumor effect of an oligonucleotide combination of CpG oligonucleotide, anti-IL-10 and anti-IL-10 receptor oligonucleotides. These nucleic acid drugs delivered by the delivery system accumulated in the TAMs of an allograft hepatoma murine model by intravenous injection, suppressed the pro-tumor functions and stimulated the anti-tumor activities of TAMs. More importantly, the nucleic acid drug-based immune-regulation was restricted to the tumor microenvironment and did not cause an upregulation of serum inflammatory cytokines. Our present study provides an effective therapeutic strategy for regulating cell-specific functions using nucleic acid drugs.

1. Introduction

Cancer immunotherapy aims to use the exquisite power and specificity of the immune system to eliminate cancer cells and reject tumors. The systemic administration of immunostimulants, such as cytokines, adjuvants and monoclonal antibodies could activate immune cells such as NK, NKT, macrophages and B cells and has yielded marked anti-tumor activity [1]. However, life-threatening toxicity often develops, largely due to non-specific stimulation of the immune system [2]. Moreover, many tumors could render anti-tumor inflammatory responses ineffective by producing large volumes of immunosuppressive cytokines such as IL-10 [3]. To improve the outcome, the regulation of immune functions should be restricted within the tumor microenvironment by intratumoral cell-specific delivery of drugs with immune regulation properties.

In the present study, we developed a tumor-associated macrophages (TAMs) targeted nucleic acid drug delivery system for use in cancer immunotherapy. An immune cell delivery system could be designed according to the properties of different cell subtypes. TAMs are a major component of leukocytic infiltration in tumors and exert various pro-tumor functions [4,5]. In this study, CpG oligodeoxynucleotide (ODN), anti-IL-10 ODN and anti-IL-10RA ODN were used in combination to alter the phenotype of TAMs and stimulate their potential tumoricidal activity. Because TAMs express high levels of macrophage galactose-type lectin (Mgl), we used galactosylated cationic dextran (gal-C-dextran), which can associate with ODN to form a stable nano-complex (GDO, gal-C-dextran + ODN), to target TAMs [6]. H^+ ions, which are generated by tumor cells in a hypoxic environment during the glycolysis process, usually accumulate in the tumors, due to insufficient drainage in the tumor tissues [7]. Therefore, tumors, especially bulky or low-flow tumors, exhibit a substantially lower intratumoral pH than normal tissues [8]. The pH-sensitive material PEG-histidine-modified alginate (PHA) was used to combine the GDO to form PDO (PHA + gal-C-dextran + ODN), which specifically releases the GDO in the acidic microenvironment of the tumor. The present study investigated the ability of PDO to target TAMs and the therapeutic effects of this targeting in a murine allograft model of liver cancer.

2. Materials and methods

2.1. Synthesis of materials and reagents

The phosphorothioate-modified CpG 1668 (5′-TCCATGACGTTCCTGATGCT-3′), anti-IL-10 ODN (5′-AGGTCCTGGAGTCCAGCA-3′) and anti-

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doi:10.1016/j.jconrel.2011.11.013

IL-10RA ODN (5′-GCCTGTGCCACGCTGCTTCC-3′) were synthesized by Invitrogen. All of the ODN sequences have been proven to be effective in previous reports and our preliminary experiments [9,10]. Mismatch control oligonucleotides, control CpG 1668 (5′-TCCATGAGGTTCCTGATGCT-3′), control anti-IL-10 ODN (5′-ATGCGCGATACGCGTAC-3′) and control anti-IL-10RA ODN (5′-CGAGTGTCCTACTGTGGTGC-3′) were used. Fluorescein isothiocyanate-labeled ODN (FITC-ODN) was synthesized for use in *in vitro* transfection. Alexa Fluor 350-labeled ODN, cyanine 5.5-labeled ODN (Cy5.5-ODN) and 6′-carboxy-2′,4,4′,5′,7,7′-hexachlorofluorescein-labeled ODN (HEX-ODN) were used in *in vivo* localization studies.

Gal-C-dextran was synthesized according to the following methods. First, cationic dextran (C-dextran) was prepared according to a previous report [11]. Next, C-dextran was coupled to lactobionic acid (LA) via an active ester intermediate using 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). Approximately 100 mg of C-dextran was dissolved in 2 ml of 10 mM *N,N,N′,N′*-tetramethylethylenediamine (TEMED)/HCl buffer solution (pH 4.7). Next, approximately 180 mg of EDC was added to this solution and stirred at 25 °C for 24 h. After that step, 2.1 mg of LA was added to the tubes of solution and stirred for another 72 h at 25 °C. The resulting gal-C-dextran was dialyzed for 4 days against double distilled water and was lyophilized. PEG-His-modified alginate (PHA) was synthesized according to a previously reported method [12]. Briefly, 500 mg of alginate (Sigma) and 1.0 g of EDC was added to the TEMED/HCl buffer and stirred at 40 °C for 24 h. Next, 1.5 g of histidine was added and the solution was stirred for another 24 h at 40 °C. His-alginate was dialyzed for 4 days against double distilled water and was lyophilized. Pegylation was achieved by conjugating a 2000 Da mPEG-NH2 to the His-alginate using a Pegylation kit (YARE Chem). FITC-labeled gal-C-dextran and rhodamine-labeled PHA were synthesized using fluorescence-label kits (DAZHI Biotech).

2.2. Tumor and mice

Hepa 1–6 (ATCC) is a mouse hepatoma cell line derived from the BW7756 mouse hepatoma that arose in a C57/L mouse. Hepa 1–6 cells were cultured in DMEM supplemented with 10% FCS. Female ICR mice (18–20 g) of the same background were purchased from the experimental animal center of Nanjing Medical University. All animals received human care according to Chinese legal requirements. To generate the allograft model of liver cancer, Hepa 1–6 cells (1×10^7 cells/ml, 10 μl) were injected into a hepatic lobe of anesthetized mice using a microinjector [13].

2.3. Preparation of GDO and PDO

GDO was obtained by mixing 5 mg/ml of ODN saline solution (Different ODNs were equal in this solution) with 5 mg/ml of gal-C-dextran solution at the volume ratio of 1:3. PDO was prepared by mixing GDO solution with the same volume of 5 mg/ml PHA solution as gal-C-dextran solution by gentle agitation for 1 h. The diameters of GDO and PDO at different pH values were analyzed by photon correlation spectroscopy using the 90 Plus Particle Sizer (Brookhaven Instruments).

2.4. Cell transfection

TAMs were isolated from the tumors, as previous described [6]. Purified macrophages were greater than 95% pure as assessed by immunofluorescence staining for F4/80. TAMs were cultured in 24-well plates for the transfection experiment. Before transfection, complete medium was removed, and cells were rinsed once with PBS. The naked ODN, C-dextran/ODN or gal-C-dextran/ODN complex containing 1 μg of FITC-labeled ODN was diluted with 0.3 ml medium and was used to refill the well. After incubation at 37 °C for 6 h, the medium containing the complex was removed. The cells were rinsed twice with PBS and refilled with medium. Transfection of the lipofectamine/ODN complex was performed as controls according to the manufacturer's protocol. The transferred cells were examined by fluorescent microscopy (TE2000-U, Nikon). To quantify the transfection efficiency, transferred TAMs were lysed with 1% TritonX-l00 (Sangon Biotech) and analyzed using a microplate reader (Tecan Group Ltd.). To investigate their inhibitory effect on cellular uptake of ODN, potential competitor solutions of lactose, N-acetyl galactosamine (GalNAc), N-acetyl glucosamine (GlcNAc), or dextran (100 mmol/L) were pre-incubated with cells for 1 h before transfection with the gal-LMWC/ODN complex.

2.5. Release of ODN from the complexes

To confirm the tumor-specific drug-release of PDO, *in vivo* experiments were performed. Alexa Fluor 350-labeled ODN, rhodamine-labeled PHA and FITC-labeled gal-C-dextran were used to form PDO. We opened the abdomen of a liver tumor-bearing mouse that was anesthetized with pentobarbital sodium (Sigma). From the morphological appearance, we could distinguish the tumor from the liver. After locating the tumor, PDO was directly injected into the tumor using a microinjector. One hour after PDO injection, the orthotopic tumor tissues were isolated from the livers of tumor-bearing mice by surgical excision. As a control, we also injected PDO into a hepatic lobe of a control mouse and excised the liver from the mouse 1 h later. The liver and tumor tissues were rapidly embedded in O.C.T. compound (Sakura), frozen sectioned and examined under a fluorescent microscope (TE2000-U).

2.6. Tissue-distribution and cellular localization of ODN

GDO, PDO and naked ODN solutions were separately injected into mice via tail vein at a dose of 1.5 mg HEX-ODN or Cy5.5-ODN/kg body weight. Different organs were harvested from the experimental mice bearing implanted tumors. Liver and tumor containing Cy5.5-ODN were imaged by IVIS Lumina XR system. HEX-ODN was used to determine the cellular localization of ODN. Frozen sections of the tumors harvested 24 h after administration of the complex and were stained with a rat anti-mouse F4/80-antibody (eBioscience) at 4 °C overnight. The secondary antibody, FITC-labeled goat anti-rat (KPL), was applied at 37 °C for 30 minutes followed by the staining of nuclei with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Frozen sections were imaged using a fluorescent microscope (TE2000-U, Nikon). For the quantification of cellular uptake, TAMs were isolated and non-adherent cells were used as non-TAM. HEX-ODN in the organs and cells was extracted according to a reported method and quantified by examining the fluorescence intensity at 556 nm [14].

2.7. Anti-cancer activity of PDO

Animals bearing implanted tumors were intravenously given GDO, PDO and naked ODN at a dose of 1.5 mg ODN/kg body weight every three days beginning on day 5 after the allograft model was established. All tumors were excised, weighed and sectioned for histopathological and immunofluorescence analysis on day 21. TAMs isolated from these tumors were used for mRNA quantification (Mgl1, Mgl2, IL-10RA, arginase 1 (Arg1), chitinase 3-like 3 (Ym1), macrophage scavenger receptor 2 (Msr2) and resistin like alpha (FIZZ1)) and analysis of secreted cytokines (IL-10, IL-12, VEGF and MMP9). Serum was also collected for ELISA assays (R&D) (IL-1β, IL-6, TNF-α, IL-12 and IL-10).

2.8. mRNA quantification

Total RNA from TAMs were prepared using TRIzol reagent (Invitrogen). Real-time qPCR was performed using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) according to the