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Feasibility of a subcutaneously administered block/homo-mixed polyplex micelle as a carrier for DNA vaccination in a mouse tumor model

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ABSTRACT

In this study, the potential of DNA vaccine by subcutaneously (s.c.) administered block/homo-mixed (B/H) polyplex micelles carrying genes encoding tumor-associated antigen SART3 as well as CD40L and GM-CSF was compared with the intraperitoneal (i.p.) and intravenous (i.v.) administrations or electroporation method. Confocal laser microscopy revealed high localization of polyplexes in groin lymph nodes and local skin tissues after s.c. administration, and in the mesenteric lymph nodes, liver, and spleen after i.p. administration, but not after i.v. administration. Real-time RT-PCR and immunohistochemistry showed transgene expression in the above organs by s.c. and i.p. administered B/H polyplex micelles, but not by the i.v. administration or electroporation. Polyplex-carried DNA vaccines significantly decreased the weight of subcutaneous CT26 tumors in mice compared to the mock (2.9 ± 0.8 vs 6.4 ± 2.6 g, $P < 0.05$ for s.c.; 3.2 ± 1.1 vs 4.7 ± 2.1 g, $P < 0.05$ for i.p. administration). The survival rate was improved by s.c. administration of the DNA vaccine ($P < 0.05$) and by the i.p. administered DNA vaccine ($P < 0.01$) compared with that of the mock controls in mice with peritoneally disseminated CT26 cancer. Such therapeutic effects were not observed by the naked DNA, i.v. administered DNA vaccine or electroporation. CTL and NK cell activities of splenocytes and infiltration of $CD11c^+$ DCs, and $CD4^+$ and $CD8a^+$ T cells into tumor tissues were upregulated in the s.c. administered DNA vaccine group ($P < 0.05$), which was consistent with i.p. administration. No abnormal findings in local injection sites, body weight, or blood examinations were observed by s.c. or i.p. administration of polyplex micelles, whereas proinflammatory cytokine production was minimized in visceral organs with the s.c. administered polyplex-carried DNA vaccine. In conclusion, s.c. administration of B/H polyplex micelles may be a safe and useful modality for DNA vaccination.

1. Introduction

The development of non-viral gene delivery systems has gained attention because of the advantages of low host immunogenicity and large-scale manufacturing [1,2] in comparison with viral vectors that have safety concerns, a limited size of the loading gene, and difficulty in large-scale manufacturing [3]. Many of synthetic gene carriers are formulated through electrostatic self-assembly of anionic plasmid DNA (pDNA) and cationic materials (e.g. cationic lipids and polymers) [4]. In addition, the conjugation of polyethyleneglycol (PEG) to cationic lipids or polymers, in which pDNA is packaged within the core and surrounded by a PEG shield, protects the genes from interactions with biological components, resulting in substantial stability within the physiological environment. For instance, a cationic polymer of poly{N'-[N-(2-aminoethyl)-2-aminoethyl] aspartamide}, P[Asp(DET)] (H) developed by Kataoka and co-colleagues has advantages of pH-responsive protonation to enhance the pDNA escape from endosome [5] and biodegradation under physiological conditions to minimized the cumulative toxicity [6] despite inflammatory cytokine production [7]. PEG-conjugated form of P[Asp(DET)], PEG-P[Asp(DET)] (B) minimizes the inflammatory response and improves the biocompatibility by PEG-shield even though the decrease in transducing ability occurs due to PEG's dilemma [8]. Cumulatively, B/H-mixed polyplex micelle, which has been optimized with B/H ratio of 70/30 in previous studies [7,9], gains more suitable properties of high transfection efficiency, stability in the physiological environment and few inflammatory reaction in vivo.

Although the transfection efficiency of non-viral gene carriers has been improved by modifications with the above combination and/or integration of moieties to increase cellular/nuclear uptake [10–14], high gene expression enough to eradicate malignancies may not be easily achieved in vivo. Therefore, one of approaches achieving therapeutic effect in vivo is to apply for immuno-gene therapy, such as DNA vaccine, to synergize anti-tumor effect by activated immune

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effectors. Transduction of tumor-associated antigen (TAA)'s pDNAs into antigen-presenting cells (APCs) plays a critical role in DNA vaccine eliciting specific rejection immunity against the tumors. Recently, we have shown that intraperitoneal (i.p.) administration of the B/H polyplex micelles delivers pDNAs to APCs in lymphatic organs [15]. Although a transduction of tumor-associated antigen of squamous cell carcinoma antigen recognized by T cells 3 (SART3) pDNA alone did not induce rejection immunity, we found that triple combination of SART3, CD40L and GM-CSF gene transduction by i.p. administered B/H polyplex micelles elicited anti-tumor vaccine effect by the activation of APCs and subsequent infiltration of helper T and cytotoxic T lymphocytes (CTL) into tumors with autologous, low-immunogenic TAA of SART3 [15].

I.p. administration of the B/H polyplex micelles is a simple but not popular procedure in clinics, and may induce the adverse events of peritoneal irritation, inflammation, and adhesion of the peritoneum in patients who have problematic general and/or abdominal conditions. Subcutaneous (s.c.) injection of peptide vaccines in the skin near the groin and axillary LNs are popularly used in clinical trials, because APCs including dendritic cells (DCs) and their subset of Langerhans cells are enriched in epidermal/dermal region of skin tissues [16,17]. In addition, transduction of TAA/adjuvant genes into skin fibroblasts is expected to elicit cross-presentation to neighboring APCs [18]. However, a simple and convenient gene delivery system via transdermal/subcutaneous routes has not met with success, although gene gun using nano-gold coating particles [19] and electroporation [20,21] have been investigated for the modality of transdermal DNA vaccination in experimental models. Alternatively, cationic liposome and polyplex have achieved sufficient transfection efficiency in vitro, but antitumor vaccine effect is not induced in tumor models with autologous TAA when administered into the skin tissues, because of electrostatic interaction between cationic carriers and negative charged extracellular matrixes [22]. We hypothesize that s.c. administration of B/H polyplex micelles, which PEG shields mask the cationic charge, may escape the trapping by extracellular matrixes and deliver vaccine DNA to APCs in the skin and regional LN tissues, resulting in anti-tumor vaccine effect. Furthermore, s.c. administration is expected to have a higher safety reserve compared with i.p. administration in recipients who have problematic conditions.

To address the hypothesis, we herein examined the distribution, gene expression, anti-tumor efficacy and safety profile of DNA vaccination with s.c. administered B/H polyplex micelles in low-immunogenic tumor models. S.c. administration of B/H polyplex micelles exhibited the same level of anti-tumor effect with less production of proinflammatory cytokines in visceral organs compared with i.p. administration, although both types of B/H polyplex micelles were superior to the electroporation method regarding the efficacy and safety potential.

2. Materials and methods

2.1. Cell lines

Murine colorectal carcinoma (CT-26) and lymphoma (YAC-1) cell lines were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI 1640 medium (Nacalai Tesque Ltd., Kyoto, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Wako Pure Chemical Industries Ltd., Osaka, Japan), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in humidified incubators containing 5% CO_2.

2.2. Animals

BALB/c AnNCrlCrlj mice (female, 6 weeks old) were purchased from Charles River Laboratories Japan (Yokohama, Japan). Animals were housed in a temperature-controlled room under 12/12 hour light/dark cycles and had access to food and water ad libitum. All animal procedures were approved and carried out in accordance with the institutional Guidelines for Animal Experiments of the Animal Care and Use Committee at Kyushu University.

2.3. Plasmid DNA construction

Expression plasmids for GM-CSF, CD40L and SART3 genes (accession numbers BC116880.1, NM_011616.2 and NM_016926.1, respectively) were constructed as reported previously [15]. The open reading frames of mouse GM-CSF, CD40L and SART3 genes were integrated at the multi-cloning sites in the pVIVO1-mcs2 plasmid (InvivoGen, Cayla, France). The pDNAs were amplified in *Escherichia coli* DH5A competent cells and purified using an EndoFree Plasmid Giga Kit (QIAGEN K.K., Tokyo, Japan).

2.4. Preparation of polyplex micelles encapsulating pDNA

Homo-catiomer poly{N′-[N-(2-aminoethyl)-2-aminoethyl] aspartamide} P[Asp(DET)] (degree of polymerization (DP): 55) and block-catiomer PEG-*b*-P[Asp(DET)] (M_w of PEG: 12000; DP: 65) were kindly provided by NOF Corp. (Kawasaki, Japan). The B/H polyplex micelles were prepared by mixing pDNA (50 μg = 25 μg for SART3 pDNA and 25 μg for CD40L/GM-CSF pDNA) solution and pre-mixed PEG-*b*-P[Asp(DET)] and P[Asp(DET)] solution in a 10 mM HEPES buffer (pH 7.3) at a block/homo charge ratio of 70/30 and N/P ratio of 10 (N = total amines in polycations; P = total phosphate anions in pDNA) [9]. After mixing the pDNA and polymers, the micelle solution was concentrated in a 10 μm-filtered centrifugation tube (7040 ×*g*) for 20 min to 0.25 mg/ml (final pDNA concentration). The ζ-potential of the B/H polyplex micelle was measured by an ELSZ-2 (Otsuka Electronics, Osaka, Japan) at 25 °C. The size and polydispersity index (PDI) of the polyplex micelle were evaluated by measurement of dynamic light scattering (DLS) at 25 °C using the ELSZ-2 analyzer with a detection angle of 160° and a He–Ne ion laser (633 nm) as the incident beam as reported previously [23].

2.5. In vivo distribution of polyplex micelles

Fluolid Orange NHS (International Science Technology Co. LTD., Fukuoka, Japan) was conjugated to the amino groups of PEG-b-P[Asp(DET)] as described previously [23]. Fluolid-labeled polyplex micelles loaded with pDNA (50 μg/mouse) were s.c., i.p. or i.v. injected into BALB/c mice. At 24 h after injection of Fluolid-labeled micelles, the groin LN, spleen, mesenteric LN, kidney, lung and liver tissues were obtained, embedded in OCT compound, and frozen. Sections (8–10 μm) were prepared, air-dried, and fixed in cold acetone for 10 min. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). The Fluolid signal of polyplexes was observed by confocal laser microscopy (NIKON $C2^+$; Nikon Instruments Co. Ltd., Tokyo, Japan).

2.6. In vivo gene transduction with polyplex micelles

We administered solutions (200 μl) of mock or vaccine (GM-CSF, CD40L, and SART3) pDNA-loaded B/H polyplex micelles (50 μg pDNA/mouse) via s.c., i.p. or i.v. routes into BALB/c mice. Injection sites for s.c., i.p. and i.v. routes were in subcutaneous regions near the groin LNs, peritoneal cavity, and tail vein, respectively.

2.7. In vivo gene transduction with an electroporation system

The pDNA was diluted in opti-MEM (50 μg in 200 μl/mouse). One-hundred microliters of the solution was injected into the subcutaneous tissues near the groin LNs of both sides or into muscle tissues of both legs, and then pulsed three times by an NEPA-21 electroporator (Nepagene, Chiba, Japan) at a voltage of 100 V and pulse interval of 3 s.