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Effective transgene expression without toxicity by intraperitoneal administration of PEG-detachable polyplex micelles in mice with peritoneal dissemination

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ABSTRACT

Block copolymer of poly(ethylene glycol)-*block*-poly{N-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} (PEG-P[Asp(DET)]) has been originally introduced as a promising gene carrier by forming a nanomicelle with plasmid DNA. In this study, the polyplex micelle of PEG-SS-P[Asp(DET)], which disulfide linkage (SS) between PEG and cationic polymer can detach the surrounding PEG chains upon intracellular reduction, was firstly evaluated with respect to *in vivo* transduction efficiency and toxicity in comparison to that of PEG-P[Asp(DET)] in peritoneally disseminated cancer model. Intraperitoneal (i.p.) administration of PEG-SS-P[Asp(DET)] polyplex micelles showed a higher ($P<0.05$) transgene expression compared with PEG-P[Asp(DET)] in tumors. In contrast, the delivered distribution of the micelles was not different between the two polyplex micelles. PEG-SS-P[Asp(DET)] micelle encapsulating human tumor necrosis factor α (hTNF-α) gene exhibits a higher antitumor efficacy against disseminated cancer compared with PEG-P[Asp(DET)] or saline control. No hepatic and renal toxicities were observed by the administration of polyplex micelles. In conclusion, PEG-detachable polyplex micelles may represent an advantage in gene transduction *in vivo* over PEG-undetachable polyplex micelles after i.p. administration for peritoneal dissemination of cancer.

1. Introduction

Gene therapy has attracted much attention as a promising modality to treat intractable diseases, such as genetic disorder, neurodegenerating disease and cancer [1,2]. Viral vectors, such as adenovirus vector, have been vigorously explored in these strategies, because the gene transfer efficiency is generally higher than nonviral gene vector. However, the clinical use of viral vectors has considerable limitations with respect to safety, and it is desirable to develop a nonviral vector. Recently, PEG-based cationic polymers (catiomers) have been expected as one of promising alternatives to viral gene vectors [3–8]. The block catiomers spontaneously associate with plasmid DNA (pDNA) to form sub-100 nm polyplex micelles with high colloidal stability by hydrophilic PEG palisade surrounding the core under physiological conditions and substantial transfection activity [9–14].

We have demonstrated that polyplex micelles formed by PEG-*block*-poly(aspartamide) copolymers carrying the *N*-(2-aminoethyl)-2-aminoethyl group in the side chain (PEG-P[Asp(DET)]) exhibit high efficiency and low cytotoxicity [15]. The polyplex micelles demonstrated appreciable gene transfer into vascular lesions in animal model [16] and bone defect in animal models [17]. To further achieve successful *in vivo* gene therapy, we have tried to modify the block copolymer, because P[Asp(DET)] homopolymer polyplexes show higher transfection efficiency than PEG-P[Asp(DET)] micelles [18] and the PEG palisade surrounding P[Asp(DET)] cores hampers the gene transfection (PEG dilemma) [19–21]. To overcome the dilemma, we have developed PEG detachable polyplex micelles with disulfide linkages between PEG and P[Asp(DET)], which are sensitive to the intracellular reducing environment [22]. This micelle showed higher gene transfection efficiency than the micelle without disulfide linkages by several orders of magnitude *in vitro* assay [22]. However, the transduction on efficacy and safety for *in vivo* application remains to be evaluated.

Systemic chemotherapy is mainly conducted for the patients bearing refractory advanced malignancies, such as liver or lung metastasis and peritoneal dissemination [23,24]. Among these metastases, peritoneal lesions exhibit relative resistance to systemic chemotherapy due to peritoneal-plasma barrier, which prevents the effective drug delivery from blood vessels into the peritoneal lesions

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[25]. In comparison with conventional systemic chemotherapy, the intraperitoneal (i.p.) administration of hydrophobic chemotherapeutic agents or vectors encapsulated therapeutic genes appears to have an advantage for peritoneal dissemination, because the drugs are directly delivered into the peritoneal cavity and retarded in tumor nodules, which enables to keep high dose concentration in tumor tissues [26]. In addition, gene therapy prevents the rapid enzymatic degradation of therapeutic molecules, such as tumor necrosis factor α (TNF-α), which is known to exhibit anti-tumor effect for a wide variety of cancers [27], and the inflammation-related toxicity induced by its systemic administration of high dose cytokines, although tumor vasculature-specific ligands could increase an accumulation of RGD4C-TNF-α into tumor vicinities [28,29].

In this study, we examined the feasibility of PEG detachable polyplex micelles on transduction efficiency, therapeutic efficacy and safety as a gene delivery system compared with the conventional polyplex without disulfide linkage. The intraperitoneal administration of PEG-detachable polyplex micelles with RGD4C-TNF-α-encoding plasmid, but not that of PEG-undetachable polyplex, revealed an appreciable *in vivo* gene expression and antitumor effect against peritoneal metastases of pancreatic cancer, despite of no difference in the preferable distribution for tumor and lymphatic tissues.

2. Materials and methods

2.1. Chemicals

α-Methoxy-ω-hydroxyl PEG (PEG-OH, $M_n = 12{,}000$, $M_w/M_n = 1.03$) β-benzyl L-aspartate *N*-carboxyanhydride (BLA-NCA) were obtained from NOF Corporation (Tokyo, Japan) and Chuo Kaseihin Co., Inc. (Tokyo, Japan). Methanol (MeOH), 2-aminoethanethiol, diethyl ether, ammonia (NH_3), hexane, ethyl acetate (AcOEt), and hydrochloric acid (HCl) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used as received. Dichloromethane (CH_2Cl_2), *N*,*N*-dimethylformamide (DMF), diethylenetriamine (DET) and *N*-methyl-2-pyrrolidone (NMP) were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) or Nacalai Tesque (Kyoto, Japan) and purified by distillation before use. Linear polyethyleneimine (LPEI, $M_w = 22{,}000$) was purchased from Polysciences, Inc. (Warrington, PA). Water was purified using a Milli-Q instrument (Millipore, Bedford, MA).

2.2. Synthesis and characterization of PEG-SS-P[Asp(DET)]

PEG-SS-P[Asp(DET)] was prepared as previously reported [22] with minor modification. Briefly, PEG-OH was converted to PEG-SH [30], of which conversion rate was estimated as 80% from ^{1}H NMR spectrum. The crude PEG-SH (0.85 g) was dissolved in MeOH (30 mL), followed by the reaction with 2-aminoethanethiol (100 equiv, 0.65 g) at room temperature to obtain PEG-SS-NH_2. The polymer components were precipitated into an excess amount of diethyl ether, and then the filtrate was dried *in vacuo*. PEG-SS-NH_2 was isolated from the crude product by passing through an ion-exchange gel (SP Sephadex C-50, GE Healthcare, Tokyo, Japan) column. Adsorbed PEG-SS-NH_2 was eluted from the gel by diluted NH_3 aqueous solution, evaporated using rotary evaporator at 30 °C, and then lyophilized as a white powder (0.63 g in yield). The functionality of the PEG end to the aminoethanethiol moiety was confirmed to be 97% by an ion-exchange HPLC equipped with TSKgel SP-5PW column (TOSOH Corporation, Tokyo, Japan).

The PEG-SS-poly(β-benzyl L-aspartate) (PEG-SS-PBLA) block copolymer was prepared by the ring opening polymerization of BLA-NCA (3.3 mmol, 0.97 g) in CH_2Cl_2/DMF (10/1 (v/v), 23 mL) at 35 °C from the terminal primary amino group of PEG-SS-NH_2 (0.04 mmol, 0.50 g). The reaction mixture was added into an excess amount of hexane/AcOEt (6/4 (v/v)), and then the filtrate was dried *in vacuo* (1.17 g in yield). The degree of polymerization (DP) of PBLA was calculated to be 69 by ^{1}H NMR spectroscopy based on the peak intensity of benzyl protons of PBLA ($-OCH_2C_6H_5$, δ = 7.3 ppm) to the ethylene protons in PEG ($-OCH_2CH_2$, δ = 3.6 ppm).

Lyophilized PEG-SS-PBLA (230 mg) was dissolved in NMP (9 mL), followed by the reaction with DET (4 mL, 50 equiv to benzyl group of PBLA segment), diluted in NMP (4 mL) under anhydrous conditions at 5 °C. After 30 min, the reaction mixture was slowly added dropwise into a 5 N HCl aqueous solution, where the temperature of the mixture was kept below 5 °C. Then the mixture was dialyzed against 0.01 N HCl and, subsequently, deionized water (MWCO: 12–14 kDa) at 4 °C. The final solution was lyophilized to obtain PEG-SS-P[Asp(DET)] (Fig. 1) as the chloride salt form (213 mg in yield). The quantitative substitution from BLA to Asp(DET) was confirmed by the peak disappearance of benzyl protons ($-OCH_2C_6H_5$, δ = 7.3 ppm) and the intensity ratio of the ethylene protons in the 1,2-diaminoethani moiety ($H_2N(CH_2)_2NH(CH_2)_2NH$-, δ = 3.4–2.8 ppm) to the methylene protons in PEG ($-OCH_2CH_2$, δ = 3.6 ppm) in the ^{1}H NMR spectrum in D_2O (data not shown).

2.3. Plasmid DNA construction

A plasmid encoding luciferase (pCpG-ΔLuc) was kindly supplied from Dr. Makiya Nishikawa (Kyoto University) [31]. As expression plasmids encoding therapeutic gene, pVIVO-RGD4C-hTNF-α and pCpG-RGD4C-hTNF-α were constructed as follows; the oligonucleotide encoding RGD4C (CDCRGDCFC) with glycine–serine linker interposed between RGD4C and matured human TNF-α (hTNF-α) sequences as a spacer was synthesized. The open-read frame of RGD4C-hTNF-α was integrated at the multi-cloning sites in the plasmid DNA of pVIVO1-mcs with hamster 78-kDa glucose-regulated protein (GRP78) promoter which yields persistent high expression within the tumor micro-environment or in the plasmid of pCpGfree-mcs to delete immunogenic CpG motifs with human elongation factor 1 alpha core promoter (Invivogen, San Diego, CA). The plasmid DNA was amplified in competent DH5R *Escherichia coli* and purified using EndoFree Plasmid Giga Kits (QIAGEN Inc., Valencia, CA). The pDNA concentration was determined by reading the absorbance at 260 nm.

2.4. Preparation of PEG-SS-P[Asp(DET)]/pDNA polyplex micelles

The PEG-SS-P[Asp(DET)] block copolymer and pDNA were separately dissolved in 10 mM Tris–HCl buffer (pH 7.4). The polymer solution was added to a 2-times-excess volume of 375 μg/mL pDNA solution (final pDNA concentration: 250 μg/mL) at N/P ratio = 10, which is a molar ratio of amine units in block catiomers to phosphate units in pDNA. The mixed solution was left at 4 °C for 15 min to form polyplex micelles and then subjected to the following experiments. Just prior to *in vivo* administration, 1/10 volume of 1.5 M NaCl solution was added to form isotonic solution. The polyplexes with PEG-P[Asp(DET)] block copolymer (*M*w of PEG: 12,000; DP of P[Asp(DET)] segment: 65) or LPEI were similarly prepared and used as controls.

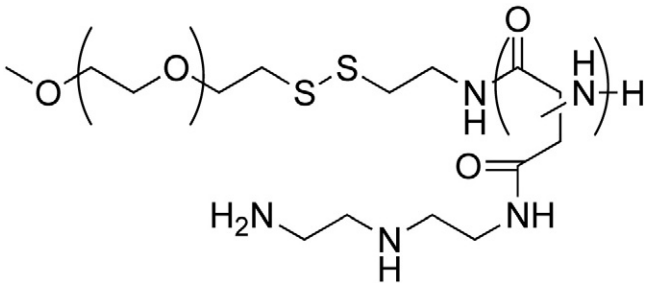

Fig. 1. Chemical structure of PEG-SS-PAsp(DET).