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DNA/polyethyleneimine/hyaluronic acid small complex particles and tumor suppression in mice

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ABSTRACT

The highest barriers for non-viral vectors to an efficient *in vivo* gene transfection would be (1) non-specific interaction with biological molecules, and (2) large size of the DNA complex particles. Protective coating of the DNA/polyethyleneimine (PEI) complexes by hyaluronic acid (HA) effectively diminished the adverse interactions with biological molecules. Here we found HA also protected the DNA/PEI complexes against aggregation and inactivation through lyophilization-and-rehydration procedures. It allows us to prepare the concentrated very small DNA complex particles (<70 nm) suspension by preparing the complexes at highly diluted conditions, followed by lyophilized-and-rehydrated to a small volume. *In vivo* gene expression efficiency of the small complex was examined with mice subcutaneously inoculated with B16 melanoma cells. These formulations showed high reporter-gene expression level in tumor after intravenous injection into tumor-bearing mice. Small complex was then made of the plasmid encoding GM-CSF gene, and injected into the mice bearing subcutaneous solid B16 tumor. After intravenous injection, it induced apparent tumor growth suppression in 50% of the mice. Notably, significant therapeutic effect was detected in the mice that received intratumoral injection, and 75% of the mice were completely cured with disappearance of tumor.

1. Introduction

Various virus vectors have been widely used in human gene therapy trials. They showed fairly high extra gene expression, and clinical effects in some cases. Safety problems, however, still exist, such as random recombination or immunogenicity [1]. Many researchers aim at preparation of safe non-viral vectors. The interests especially focus on the polycations and cationic lipids which electrostatically bind to DNA and facilitate gene transfer into target cells. Some of these transfection-mediating reagents have already realized relatively high gene expression on the cultured cells [2–4], but it still remains challenging to achieve high extragene expression levels in *in vivo* transfection by the non-viral vectors.

One of the highest barriers for the non-viral vectors to an efficient *in vivo* gene transfection should be low deliverability of the DNA complexes to the target cells after administration. Low delivery efficiency of the DNA/cation complexes would be mainly attributed to (1) their non-specific interaction with biological molecules, and (2) large size of the complex particles. Surface of the complexes are usually positively charged, and their interaction with blood calls, serum proteins and extracellular matrices prevents their efficient delivery [5–7]. Shielding of the charge by grafting the neutral polymer on the complex surface was employed to restrain the undesirable interactions, and enhanced the systemic duration [8,9]. Protective polyanion-coating was developed to afford negatively charged DNA complexes [10,11], which showed diminished interaction with serum proteins. We have also devised plasmid/polycation/polyanion ternary complex systems employing novel PEG derivatives having carboxylic acid side chain [12,13], or hyaluronic acid (HA) [14,15] as an anionic component. The resulting complexes had negative surface charge, and they expressed high dispersion stability in the presence of blood cells or proteins. The polyanion could play a role as not only protective coating, but as a ligand to target cells [7,14], and also a transcriptional enhancer [14,16], and fairly improved reporter-gene expression in tumor was practically observed after their injection into mice tail vein [13,17].

Although evident progress was made on the transfection in animals, the efficiency is still not satisfactorily high. Most serious problem remained unsolved which causes the low transfection efficiency in living body would be the difficulty in achieving and maintaining the small size of the DNA complexes. Particle with

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diameter less than 100 nm is required to be distributable in the body and accumulate in tumor by enhanced permeability and retention (EPR) effect. For *in vivo* transfection, highly concentrated DNA complex suspension ([DNA] > 150 μg/ml) is required to administer an adequate dose of DNA. Mixing of DNA and polycation under such highly concentrated conditions, however, usually gives much larger particles (>200 nm). Moreover, DNA/polycation binary complex easily aggregates to increase its size [18].

Small particles can be obtained by mixing DNA with the polycation only at very low concentration. It should then be concentrated prior to an injection. But, condensation of DNA/polycation (or cationic lipids) without inactivation is difficult. Centrifugation, ultra-filtration, or evaporation would always cause the particle aggregation. Freeze-drying of the DNA/polycation complex is also known to induce strong aggregation, and result in a significant loss of transfection efficiency [19,20]. On the other hand, polyanion-coated DNA/polycation ternary complexes are very stably dispersed [6]. They were, thus, expected to be lyophilized without aggregation or inactivation, and be able to be concentrated by freeze-drying and rehydration process.

In this study, concentrated suspension of very fine DNA complex particles coated by hyaluronic acid was prepared by mixing at low concentration, followed by lyophilizing-rehydrating condensation. Their high *in vivo* gene expression, and therapeutic effects on tumor-bearing mice were examined.

2. Materials and methods

2.1. Materials and animals

Hyaluronic acid sodium salt (from Microorganism) was obtained from Nacalai Tesque, Inc. Linear PEI (MW 25,000) and YOYO-1 iodide (YOYO-1) were purchased from Polyscience, Inc. and Invitrogen Corp., respectively. GFP-coding plasmid with cytomegavirus promoter was obtained from CLONTECH Laboratories, Inc. That with luciferase gene was constructed by subcloning the HindIII/XbaI firefly luciferase cDNA fragment from the pGL3-control vector (Promega, Madison, WI, USA) into the polylinker of the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA) [21]. Plasmid coding mouse granulocyte macrophage-colony stimulating factor (GM-CSF) was similarly produced with pcDNA3.1 vector and the gene isolated from mouse lymphocyte. They were amplified in *Escherichia coli*, and purified with a QIAGEN Plasmid Mega Kit. Cell culture lysis reagent and luciferase assay substrate were purchased from Promega Corp. Protein assay kit was obtained from Bio-Rad Laboratories. ddY mouse, a closed colony established at National Institute of Biomedical Innovation, was supplied from Tokyo Laboratory Animals Science Co., Ltd.

2.2. Preparation of DNA complex

2.2.1. Fresh DNA complex

To a solution of plasmid DNA (800 μg/ml) were added a double volume of HA solution (2.92–8.75 mg/ml) and an equal volume of linear PEI solution (1250 μg/ml) in this order. All the solutions were prepared in water or 5% glucose.

2.2.2. Freeze-dried and rehydrated DNA complex

Typically; an aqueous solution of plasmid DNA (12.5 μl, 800 μg/ml) was diluted by 0, 100, 400, or 1600 μl of water, mixed with solution of HA (25 μl; 5.84 mg/ml) and then with linear PEI solution (12.5 μl; 1250 μg/ml). All the solutions were prepared in pure water. After standing for 30 min, they were freeze-dried, and the resulting white spongy complexes were rehydrate with water or 5% glucose to be [DNA] = 200 μg/ml.

2.3. Microscopic observation of DNA complex

Fluorescence microscopic observation was performed with IX70 microscopy (Olympus) equipped with a 100× oil-immersion objective lens and a high-sensitivity Hamamatsu SIT TV camera. Plasmid, pCMVluc, was visualized with a fluorescent dye, YOYO-1. All the observation was carried out in water. Final concentration of DNA, YOYO-1 was 6.1 μg/ml and 3 μM, respectively.

2.4. ζ-Potential and size measurement

The freeze-dried complex containing 1.2 μg of DNA was rehydrate with water (6 μl). After 30 min, it was diluted with 800 μl of water and supplied to a particle analyzer (MALVERN Zetasizer Nano ZS). Freshly prepared complex was similarly diluted and analyzed in water.

2.5. In vitro transfection

B16 cells, a mouse melanoma cell line, were seeded onto 24-well plates at 1×10^5 cells per well, and cultured for 2 days in Eagle's Minimum Essential Medium (EMEM) media supplemented with 10% fetal bovine serum (FBS), penicillin G sodium (100 unit/ml), and streptomycin sulfate (0.1 mg/ml). The primary growth medium was then replaced with 500 μl of fresh EMEM with FBS and the antibiotics. Fresh or lyophilized-and-rehydrated DNA complex suspensions were prepared in pure water ([DNA] = 200 μg/ml), and diluted with the same volume of double-concentrated PBS. After 30 min, they were added to the cells (1.25 μg of plasmid per well), and incubated for 4 h at 37 °C. Fresh medium (500 μl) was then added to the wells. After an additional 20-h incubation at 37 °C, the cells were lysed, and transgene expression and protein content in the lysate were assessed with the corresponding assay kit.

2.6. In vivo transfection by the freeze-dried DNA complex

Male ddY mice (5 weeks) were inoculated subcutaneously with 8×10^6 B16 cells. When the size of the tumor reached 8–10 mm in diameter, fresh or lyophilized-and-rehydrated complex suspension containing 50 μg of plasmid (in 250 μl of 5% glucose) was intratumorally or intravenously injected. After 24 h, the mice were sacrificed, and the tissue samples were taken out. For luciferase assay, they were homogenized in 1 ml of lysis-buffer, and centrifuged. The supernatant was assayed for luciferase activity and protein content. For GFP observation, the 20 μm frozen tissue sections fixed with paraformaldehyde were prepared, and observed for the fluorescent protein expression with fluorescence microscopy (Ex = 470–490 nm).

2.7. Gene therapy on the tumor-bearing mice

An aqueous solution of plasmid coding mouse GM-CSF (100 μg in 9585 μl water) was mixed with solution of HA (1450 μg in 290 μl water) and then with linear PEI solution (294 μg in 125 μl water). After standing for 30 min, it was freeze-dried, and the resulting white spongy complex was rehydrated with 150 μl or 200 μl of PBS for intravenous or intratumoral injection, respectively. B16 cells were inoculated into the subcutaneously of male 5-weeks-old ddY mice (4×10^5 cells per mouse). When the size of the tumor reached to 3–5 mm in diameter, the re-suspended complex containing 100 μg of the plasmid was injected five times every other day, and the size of the tumor was measured every day for 35 days. The care and use of laboratory animals followed the guidelines for animal experiments of the institutes.

3. Results

3.1. The effect of HA on lyophilization-induced gene inactivation

Mixing of the plasmid with PEI and HA gave stable ternary complexes [14,15,17]. The ternary complex prepared at the mixing ratio of 1:12:12 (in charge) showed highly negative surface charge (−40 to −48 mV), regardless of the mixing order, and diminished interaction with serum proteins [14] or blood cells [17]. Protecting property of HA against gene inactivation by freeze-drying was investigated. DNA complexes were prepared at [DNA] = 6.1 μg/ml with or without HA. They were frozen-and-thawed, or lyophilized-and-rehydrated, and observed by fluorescence microscopy in each step. Both freshly prepared DNA/PEI- and DNA/PEI/HA-complexes were observed as fluctuating small particles. After freezing, the binary complex formed large aggregates, which remained undissociated as a large precipitate after thawing (Fig. 1). When the binary complex was freeze-dried on a glass slide, it caked on the glass surface, and could not be re-suspended by water. After vigorous pipetting, a few large cakes peeled off. On the other hand, in the frozen suspension of the ternary complex with HA, small dots were still observed in the frozen water. After thawing, the particles again began to fluctuate as small spots similarly as the freshly prepared complexes. After freeze-drying the ternary complex, spongy solid was obtained. On rehydration, the sponge was soon swollen, and fine particles began to vibrate in the matrix. When it was pipetted, HA matrix was dissolved and the complexes were re-suspended in water as very fine particles (Fig. 1).

Gene expression efficiency of the freeze-dried DNA/polycation complexes with or without HA was then examined on cultured B16 cells. DNA complexes were prepared at [DNA] = 200 μg/ml in final,