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Hyaluronic acid controls the uptake pathway and intracellular trafficking of an octaarginine-modified gene vector in CD44 positive- and CD44 negative-cells

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ABSTRACT

The cellular uptake pathway for a gene vector is an important factor in transgene expression. We previously constructed an original gene vector, multifunctional envelope-type nano device (MEND). The use of octaarginine (R8), a cell-penetrating peptide dramatically enhanced the transfection activity of the MEND since efficient cellular uptake via macropinocytosis, while the R8 should overcome its poor cell selectivity. Here we prepared an R8-MEND equipped with GALA (a peptide for endosomal escape) (R8/GALA-MEND) coated with hyaluronic acid (HA) (HA-R8/GALA-MEND), a natural ligand for cancer cells overexpressing CD44. We investigated the cellular uptake pathway of the HA-R8/GALA-MEND and the R8/GALA-MEND using HCT116 cells overexpressing CD44. Both carriers were taken up by cells mainly via macropinocytosis, whereas only the HA-R8/GALA-MEND was partially internalized into cells via a CD44-mediated pathway. Investigation of transgene expression showed that the HA-R8/GALA-MEND had a high transfection activity in HCT116 cells via both macropinocytotic and CD44-mediated pathways. On the other hand, the value for the HA-R8/GALA-MEND was significantly decreased compared with the value for the R8/GALA-MEND in NIH3T3 cells (CD44-negative cells). These findings indicate that the HA-coating controls the intracellular pathway for R8-modified nanocarriers, and that a CD44-mediated pathway is an important route for transgene expression.

1. Introduction

The cellular uptake pathway of a gene vector is a major factor in transgene expression [1–5]. We previously reported that carriers with a high-density of octaarginine (R8), an artificially designed cell penetrating peptide [6,7], were efficiently internalized by cells primarily via macropinocytosis rather than clathrin-mediated endocytosis, as is the case for cationic liposomes (LPs) [8]. We also developed an R8-modified gene vector, a multifunctional envelope-type nano device (MEND), consisting of a condensed plasmid DNA (pDNA) core and lipid envelopes [9,10]. The integration of R8 into the MEND (R8-MEND) dramatically enhanced the transfection activity of the MEND, approaching values as high as that for adenovirus [9,11], while the R8 needs to overcome its poor cell selectivity, since it facilitates the cellular uptake nonspecifically regardless of the cell type.

To date, our group has reported that a dual-ligand liposomal system comprised of a specific ligand and a cell-penetrating peptide (CPP) enhanced both selectivity and cellular uptake efficiency [12,13]. Takara et al. prepared dual-ligand PEGylated liposomes by modifying the end of the polyethylene-glycol (PEG) with an NGR (Asn-Gly-Arg) tumor neovasculature-homing motif peptide, which recognizes CD13, a marker for tumor endothelial cells, followed by coating the surface of the liposomes with CPP [12]. The dual-ligand system stimulated the uptake of the carriers by CD13 positive cells, synergistically [12]. Kibria et al. also reported that dual-ligand PEGylated R8-modifeid liposomes with the cyclic RGD (Arg-Gly-Asp) peptide, a specific ligand for Integrin $\alpha_v\beta_3$, showed an enhanced cellular uptake as well as a higher transfection efficiency in Integrin $\alpha_v\beta_3$ expressing cells [13]. These reports indicate that a dual-ligand liposomal system can be a useful strategy for achieving cell selective targeting with a high transgene expression.

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More recently, we developed a dual-ligand positively charged R8-MEND that was modified with negatively charged hyaluronic acid (HA) via electrostatic interactions but not with PEG [14]. HA is a natural ligand for cancer cells and liver endothelial cells over-expressing CD44, thus it would be expected that HA would be a specific ligand for/targeted to cancer and liver endothelial cells. In that study, we investigated the transgene expression of an R8-MEND modified with HA in liver endothelial cells (liver ECs) (CD44 positive cells), and determined the optimal composition of MEND needed for efficient transgene expression in liver ECs, which possesses an HA-coated lipid envelope modified with the R8 [6,7] and GALA, a pH-sensitive fusogenic peptide for efficient endosomal escape [15,16] (HA-R8/GALA-MEND).

The focus of this study was on the mechanism responsible for the cellular uptake pathway and transgene expression of the HA-R8/GALA-MEND. A series of R8/GALA-MENDs coated with various concentrations of HA (600 kDa or 80 kDa) were prepared and the physicochemical properties and the transfection activity of these preparations in HCT116 cells overexpressing CD44 were evaluated. We then investigated the cellular uptake pathway of the HA-R8/GALA-MEND and the R8-MEND using HCT116 cells, and determined the optimal composition of HA-R8/GALA-MEND required for efficient cellular uptake via a CD44-mediated pathway. Moreover, we compared the cellular uptake pathway and transfection activity of HA-R8/GALA-MEND between HCT116 cells and NIH3T3 cells (CD44-negative cells). Based on these results, we analyzed the relationship between the cellular uptake pathway and transgene expression in HCT116 cells and NIH3T3 cells. A model for transgene expression via a CD44-mediated pathway is proposed in an attempt to understand the intracellular fate of HA-coated gene vectors.

2. Materials and methods

2.1. Materials

The pcDNA3.1 (+)-luc plasmid was constructed by inserting the firefly luciferase gene (*Hind*III–*Xba*I fragment) of the pGL3-Control plasmid (Promega, Madison, WI, USA) into the pcDNA 3.1 (+) plasmid containing cytomegalovirus promoter (Invitrogen, Carlsbad, CA, USA) pretreated with the same restriction enzymes. The pDNA was purified using a Qiagen EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Amiloride and cholesteryl hemisuccinate (CHEMS) was purchased from Sigma (St. Louis, MO, USA). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was purchased from AVANTI Polar Lipids Inc. (Alabaster, AL, USA). Stearyl octaarginine (STR-R8) [6,17] and cholesteryl GALA peptide (Cho-GALA) [15,16] was obtained from Kurabo Industries, Ltd. (Osaka, Japan). Protamine was purchased from CALBIO CHEM (Darmstadt, Germany). HA (average MW 50–110 kDa, HA (80 kDa); average MW 500–700 kDa, HA (600 kDa)) was obtained from Food Chemifa (Tokyo, Japan). Hoechst 33342 was purchased by DOJINDO Laboratories (Kumamoto, Japan). Fc Receptor Saturation Reagent was purchased from Beckman Coulter, Inc. (Fullerton, CA, USA). Fluorescein isothiocyanate (FITC)-labeled CD44-antibody was purchased from Abcam (Cambridge, United Kingdom). Cy5 labeled pDNA (Cy5-pDNA) was prepared using a *Label* IT® Cy™ 5 Labeling Kit (Takara Bio INC, Shiga, Japan), essentially according to the supplier's instructions. All other chemicals were commercially available, reagent-grade products.

2.2. Cell lines and cell cultures

Human colorectal carcinoma HCT116 cells and mouse embryonic fibroblast NIH3T3 cells were purchased from ATCC (Manassas, VA, USA). HCT116 cells were maintained in McCoy's 5A modified medium (ATCC) with 10% fetal bovine serum (FBS, HyClone; Thermo Fisher Scientific K.K., Waltham, MA, USA), penicillin (100 units/mL) and streptomycin (100 μg). NIH3T3 cells were maintained in Dulbecco's modified Eagle medium (Invitrogen) with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg). These cells were cultured under an atmosphere of 5% CO_2/air at 37°C. In this experiment, an HCT116 cell line and an NIH3T3 cell line were used as a CD44-positive and CD44-negative cells, respectively.

2.3. Preparation of R8/GALA-MEND and coating the carrier with HA

The R8/GALA-MEND was prepared by the lipid film hydration method, as previously reported [9,14]. First, pDNA was condensed with polycations to form condensed pDNA particles. The pDNA (0.1 mg/mL) and protamine solutions were mixed in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) under vortexing at a nitrogen/phosphate (N/P) ratio of 2.2. Next, lipid films were produced on the bottom of a glass tube by the evaporation of a chloroform solution containing 138 nmol of lipids (DOPE/CHEMS/Chol-GALA = 9:2:0.2 (molar ratio)). Next, 250 μL of the condensed pDNA particle solution was applied to the lipid film, followed by incubation for 15 min at room temperature to hydrate the lipids. The lipid film was then sonicated for approximately 1 min in a bath-type sonicator (85 W, Aiwa Co., Tokyo, Japan) to produce GALA-MEND. A solution of STR-R8 (10 mol % lipids) was then incubated with the GALA-MEND for 30 min at room temperature to produce R8/GALA-MEND. To coat the R8/GALA-MEND with HA via electrostatic interaction, a solution of HA was added to the R8/GALA-MEND suspensions, and then incubated for 30 min at room temperature (HA-R8/GALA-MEND). To prepare MEND encapsulating Cy5-pDNA, condensed pDNA particles were prepared using Cy5-pDNA as a tracer (3% of total pDNA).

2.4. Preparation of florescent labeled HA-coated R8/GALA-modified liposomes

To observe the intracellular trafficking of the HA-coated nanocarriers, fluorescent labeled LPs were constructed, as described below. LPs containing rhodamine labeled DOPE (AVANTI Polar Lipids Inc.) were prepared by the lipid film hydration method. Lipid films were produced on the bottom of a glass tube by the evaporation of a chloroform solution containing 138 nmol lipids [DOPE/CHEMS/Cho-GALA/rhodamine labeled DOPE = 9: 2: 0.2: 0.005 (molar ratio)]. Next, 250 μL of 10 mM HEPES buffer (pH 7.4) was applied to the lipid film, followed by incubation for 15 min at room temperature to hydrate the lipids. The lipid film was then sonicated for approximately 1 min in a bath-type sonicator. The resulting suspension was then incubated with a solution of STR-R8 (10 mol% lipids) for 30 min at room temperature to produce R8/GALA-LPs. To coat with HA via electrostatic interactions, a solution of the fluorescein labeled HA (average MW 100–300 kDa; PG Research, Tokyo, Japan) was added to R8/GALA-LPs suspensions (60 g HA/mol lipid), and the resulting mixture was then incubated for 30 min at room temperature (HA-R8/GALA-LP).

2.5. Characterization of prepared carriers

Dynamic light scattering was employed to determine the hydrodynamic diameters and polydispersity index (PDI) of the nanoparticles (Zetasizer Nano ZS; Malvern Instruments, Herrenberg, Germany). The mean diameter was calculated from a cumulants analysis based on the intensity of the scattered light. We also measured the ζ potentials using Laser doppler velocimetry (Zetasizer Nano ZS).

2.6. Transfection and evaluation of transgene expression

Transfection activity was assessed by measurement of luciferase activity described below. Cells (1×10^4 cells/well) were seeded on a 96-well plate (greiner bio-one; Frickenhausen, Germany) with complete medium, under an atmosphere of 5% CO_2/air at 37°C for 24 h. After the cells were washed with phosphate-buffered saline (PBS (−)), samples containing pDNA suspended in 70 μL of serum-free medium were added to the cells. After incubation under 5% CO_2 at 37°C for 3 h, the cells were washed with PBS (−), and then further incubated for 21 h in fresh complete medium. The cells were then washed with PBS (−), and luciferase activity (relative light unit (RLU)) was measured using a Luciferase Assay System with a Reporter Lysis Buffer kit (Promega; Madison, WI, USA) by means of a EnSpire™ 2300 Multilabel Reader (PerkinElmer, Inc.; Waltham, MA, USA).

When the CD44-mediated pathway was inhibited, an FITC-labeled CD44-antibody was added to the cells 1 h before incubation with the carriers. To investigate the effect of CD44-mediated pathway on transgene expression, the relative gene expression as a percent of the transfection activity in the absence of inhibitor was calculated as follows:

Relative gene expression (%) = transfection activity with inhibitor (RLU/well)/transfection activity without inhibitor (RLU/well) x 100 (1)

2.7. Quantification of the cellular uptake of the carriers by flow cytometry

Cells (1×10^5 cells) were seeded on a 12-well plate (BD Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) with complete medium, under an atmosphere of 5% CO_2/air at 37°C for 24 h. The cells were washed with PBS (−) before incubation with the HA-R8/GALA-MEND or R8/GALA-MEND encapsulating Cy5-pDNA (total dose of pDNA, 1 μg). The cells were then atmosphere of 5% CO_2/air at 37°C. After removing the medium, the cells were washed once with ice-cold PBS (−). The cells were then collected, suspended in complete medium, isolated by centrifugation (1000 *g*, 4°C, 5 min) and washed with FACS buffer (0.5% bovine serum albumin and 0.1% NaN_3 in PBS (−)). After resuspension in 0.5 mL of FACS buffer, the cell suspension was filtered through a nylon mesh to remove cell aggregates and dust. The cells were then analyzed by flow cytometry (FACScan; Becton Dickinson). The cells were excited with a 635 nm light from an LD laser for detecting Cy5-pDNA. The fluorescence detection channel was set to the FL4 filter for Cy5-pDNA. The cellular uptake of the carriers was expressed as the mean fluorescence intensity, calculated using the CellQuest software (Becton Dickinson).

These experiments were performed in the presence or absence of an inhibitor. To inhibit CD44-mediated uptake, an FITC-labeled CD44-antibody was added to the cells 1 h before incubation with the carriers. While, amiloride was used as a macropinocytic uptake inhibitor, and it was added to cells 30 min before with the carriers.