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Original Article

Kidney-selective gene transfection using anionic bubble lipopolyplexes with renal ultrasound irradiation in mice

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Abstract

This study assessed the ability of a new ultrasound (US) responsive gene delivery carrier, bubble lipopolyplexes, to deliver genes to the kidneys. The bubble lipopolyplexes showed highly selective gene expression in kidney tubules, but only after renal irradiation with US. These bubble lipopolyplexes, however, did not increase the expression of biomarkers of kidney injury, including blood urea nitrogen, serum creatinine, kidney injury molecule-1 mRNA, and clusterin mRNA, or induce any histopathological abnormalities in the kidney. Furthermore, pDNA containing CMV early enhancer/chicken beta-actin promoter prolonged gene expression by the bubble lipopolyplexes in the kidney for 42 days. This novel renal gene delivery method, in which transfection of bubble lipopolyplexes was followed by US irradiation of the kidneys, resulting in cell-selective, high, and long-term gene expression without renal injury in mice, may have future applications in patient treatment.

Key words: Gene delivery; Kidney; Sonoporation; Liposomes

Background

The kidneys are essential organs, being involved in the excretion of wastes, the regulation of electrolytes, the maintenance of acid–base balance, and the regulation of blood pressure. Therapeutic agents, however, have relatively limited effects in the kidneys. Methods are therefore underway to develop gene therapy methods targeting the kidneys, thus enabling the treatment of various kidney diseases, including Alport syndrome, glomerulonephritis, diabetic nephropathy, and renal fibrosis.[1–4] To be effective, these gene delivery methods must allow long-term, cell-specific expression of transgenes.[5] To date, several such renal gene delivery methods have been developed, using adeno-associated virus, a hemagglutinating virus of Japan (HVJ)–liposome system, polyethylenimine, cationic liposomes, and naked plasmid DNA (pDNA).[6–12]

Recent findings have suggested that microbubbles and/or bubble liposomes containing ultrasound (US) imaging gas followed by exposure to US could deliver pDNA directly into the cytosol without endocytosis and result in high levels of gene expression.[13–16] Efficient gene expression, however, requires the control of biodistribution between the pDNA and the microbubbles or bubble liposomes. We therefore developed US

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responsive carriers, consisting of nucleic acids complexed with cationic bubble liposomes (bubble lipoplexes) or mannosylated bubble lipoplexes, for delivery of pDNA and/or small interfering RNA (siRNA).[17,18] Both types of bubble lipoplexes were cationic, forming stable complexes via electrostatic interactions. To be utilized clinically, the surface charge of bubble lipoplexes must be anionic or neutral because cationic lipoplexes and polyplexes tend to interact with anionically charged serum albumin and erythrocytes, resulting in aggregation.[19,20] We have therefore developed anionically charged US-responsive bubble lipopolyplexes, consisting of ternary complexes of pDNA, cationic protamine sulfate, and anionic bubble liposomes, for efficient and safe transfection.[21] These anionic bubble lipopolyplexes showed low degrees of aggregation with erythrocytes and hemolysis. Transfection of these bubble lipopolyplexes into mice, followed by US irradiation of the abdominal area, resulted in high gene expression in the liver without hepatic toxicity or the production of proinflammatory cytokines.

Since these bubble lipopolyplexes are anionic, their transfection, followed by US irradiation of the kidneys, can result in efficient, organ-selective gene expression in the kidneys using a minimally invasive procedure. Although co-administration of naked pDNA with microbubbles[22] or bubble liposomes[23] has resulted in renal gene delivery, the use of bubble lipopolyplexes followed by renal US irradiation has not been well documented. Furthermore, the treatment of hereditary and chronic diseases requires sustained gene expression. Several promoters, including hepatitis B virus, albumin, and CMV early enhancer/chicken beta actin (CAG) promoters, have been reported to achieve long-term gene expression in the liver.[24–26] Although these promoters may also result in sustained gene expression in the kidneys, methods for renal transfection of genes under the control of a CAG promoter, in complex with bubble lipopolyplexes, followed by renal US irradiation, have not yet been developed.

This study was therefore designed to investigate the ability of anionic bubble lipopolyplexes to optimize gene transfection into kidneys. Bubble lipopolyplexes containing genes under the control of a CAG promoter were intravenously injected into mice, followed by renal US irradiation. Factors evaluated included the identity of cells transfected, the efficiency of transfection, and renal injury.

Methods

Chemicals

Protamine sulfate (PS) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Distearoyl phosphatidylglycerol (DSPG) and distearoyl phosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Methoxy-polyethyleneglycol 2000-distearoyl phosphatidylethanolamine (PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). All other reagents were of the highest grade of purity.

Construction of pDNA and preparation of bubble lipopolyplexes

The *Hin*d III/*Xba* I fragment of firefly luciferase cDNA was excised from the pGL3-control vector (Promega, Madison, WI, USA) and cloned into the polylinker of the vector pCAG-Neo (Wako Pure Chemical Industries Ltd., Osaka, Japan), which had been treated with *Hin*d III and *Spe* I, yielding the plasmid pCAG-Luc. The construction of pCMV-Luc has been described.[27] The pDNA was amplified using an EndoFree Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) and dissolved in sterile dH_2O. The bubble lipopolyplexes were prepared as previously reported.[21] Briefly, 250 μg of pDNA was mixed with 312.5 μg of PS, followed by 625 μg of anionic liposomes (AL) containing DSPG, DSPC, and PEG-DSPE at a molar ratio of 7:2:1, respectively. The solution containing these pDNA/PS/AL complexes was diluted to 2 mL in 5 mL sterilized vials, which were subsequently capped and pressurized with 7.5 mL of perfluoropropane gas (Takachiho Chemical Industries Co., Ltd., Tokyo, Japan). To encapsulate the US imaging gas within the anionic lipopolyplexes, the vials were sonicated in a bath-type sonicator (AS ONE Co., Osaka, Japan) for 5 min.

Animals

All animal experiments in this study were performed in accordance with institutional guidelines and were approved by the Kyoto University Animal Care Committee. Female ICR mice (5 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and acclimated to their environment for at least 1 day.

In vivo gene expression

The mice were anesthetized and fixed onto a stainless steel plate. Their abdominal hair was removed, and a US contact gel was placed on their skin. A 20 mm US probe was applied to the abdomen of each mouse. Using a 26-gauge needle, 50, 100, 200, and 400 μL of bubble lipopolyplex solution containing 125 μg of pDNA in 1 mL was injected into the tail vein of each mouse. Immediately afterward, a Sonopore-4000 sonicator (Nepa Gene Co., Ltd., Chiba, Japan) was used to apply US (frequency, 1.0 MHz; duty, 50%; burst rate, 10 Hz) transdermally to the hypogastric area, at intensities of 0.125, 0.25, 0.5, and 1 W/cm^2 (spatial average temporal peak, SATP) and durations of 0, 5, 10, 15, 30, and 60 s. Six hours later, the mice were sacrificed, and their organs including the liver, kidneys, spleen, heart, and lungs were collected. These organs were washed twice with cold saline and homogenized in lysis buffer (0.05% Triton X-100, 2 mM EDTA, 0.1 M Tris; pH 7.8) at ratios of 5 mL/g for the liver and 4 mL/g for all other organs. The homogenates were centrifuged at 21,500 × g for 10 min at 4 °C. The luciferase activities of the resulting supernatants were determined using a PicaGene Luminescence Kit (Toyo Ink Co., Ltd., Tokyo, Japan) and a luminometer (Lumat LB 9507, EG&G Berthold, Bad Wildbad, Germany) and normalized to protein content measured using a Protein Quantification Kit (Dojindo Molecular Technologies, Inc., Tokyo, Japan). The luciferase activity (pg/mg protein) of each preparation was calculated by comparison with a standard curve constructed using six serial dilutions of luciferase standard solution. For *in vivo* imaging, the anesthetized mice were injected intraperitoneally with 10 mg D-luciferin (Promega) in 1 mL phosphate-buffered saline (PBS) 6 h after transfection. Ten minutes later, luminescent images were taken using a NightOWL LB 981 NC instrument (Berthold Technologies). The pseudocolor luminescent images were overlaid with organ images and luminescence in organs was determined using WinLight software (Berthold Technologies).