An Approach to Transgene Expression in Liver Endothelial Cells Using a Liposome-Based Gene Vector Coated with Hyaluronic Acid

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ABSTRACT: Dysfunctional sinusoidal liver endothelial cells (LECs) are associated with liver diseases, such as liver fibrosis, cirrhosis, and portal hypertension. Because of this, gene therapy targeted to LECs would be a useful and productive strategy for directly treating these diseases at the level of genes. Here, we report on the development of a transgene vector that specifically targets LECs. The vector is a liposome-based gene vector coated with hyaluronic acid (HA). HA is a natural ligand for LECs and confers desirable properties on particles, rendering them biodegradable, biocompatible, and nonimmunogenic. In this study, we constructed HA-modified carriers, and evaluated cellular uptake and transfection activity using cultured LECs from KSN nude mice (KSN–LECs). Cellular uptake analyses showed that KSN–LECs recognized the HA-modified carriers more effectively than skin endothelial cells. The transfection assay indicated that the efficient gene expression in KSN–LECs, using the HA-modified carriers, required an adequate lipid composition and a functional device to control intracellular trafficking. This finding contributes to our overall knowledge of transgene expression targeted to LECs. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:3119–3127, 2013

Keywords: hyaluronic acid; liver endothelial cell; multifunctional envelope-type nano device; transgene expression; non-viral gene vector; DNA delivery; liposomes; nanotechnology; plasmid DNA; transfection

INTRODUCTION

Dysfunction of the liver appears to be associated with the development of various diseases because the organ possesses various functions including glycogen storage, hormone production, and clearing toxic molecules from the body. It has recently been demonstrated that a functional defect in sinusoidal liver endothelial cells (LECs) is associated with liver fibrosis, cirrhosis, and portal hypertension because the sinusoidal vascular network is disrupted.[1] It has also been reported that the defenestration of LECs leads to the development of hyperlipidemia because it is difficult for lipoproteins to reach hepatocytes, which are the primary cells involved in lipoprotein uptake.[2] Thus, an effective drug delivery system would be highly desirable in terms of the treatment of these LECs-related diseases.

Hyaluronic acid (HA) is a naturally occurring, biodegradable, biocompatible, and nonimmunogenic anionic biopolymer and presents a wide range of animal tissues from vertebrates to bacteria.[3] When exogenous HAs are injected intravenously, these are rapidly removed from the blood and mainly accumulate in LECs[4,5] because these cells express HA receptors. To date, HA has been widely used as a targeted delivery material for LECs. Polycations, such as poly-L-lysine[6] and polyethyleneimine,[7,8] were

Additional Supporting Information may be found in the online version of this article. Supporting Information

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covalently conjugated with HA, and these materials enabled the target-specific delivery of HA to receptor positive cells *in vitro*[7,8] and to LECs *in vivo*.[6] We also reported that cationic liposomes modified with HA–stearylamine effectively accumulate in the liver when intravenously injected, whereas accumulation in the lung was drastically reduced compared with nonmodified liposomes.[9] Moreover, fluorescent microscopy observations showed that HA-modified liposomes preferentially accumulated in close proximity to blood vessels compared with nonmodified liposomes, suggesting that the HA-modified liposomes are ultimately distributed in LECs.

However, gene expression using an HA-coated gene vector was not investigated, although the HA-modified liposomes were delivered to LECs *in vivo*. To achieve gene expression using a gene vector, after reaching the LECs, various phases of intracellular trafficking, including cellular uptake, endosomal escape, and nuclear, must be regulated. We were concerned that a gene vector with an anionic HA coating might have a low transfection activity because, generally, the transfection activity of anionic carriers is lower than that of cationic carriers, as control of the intracellular trafficking was difficult. Thus, we concluded that investigating gene expression in LECs using the HA-coated gene vector is an important issue in terms of optimizing transfection activity.

The objective of this study is to develop a liposome-based gene vector coated with HA for the efficient transgene expression in the LECs. The characteristics of carriers used in this study are summarized in Table 1. We first investigated the cellular uptakes of carriers by cultured LECs (KSN–LECs) to validate whether this cell line is optimal model to evaluate transgene expression in the LECs via HA receptor. In this experiment, we prepared HA-conjugated polyethylene glycol liposome (HA–PEG–LP), where *PEG* is present on the carrier surface as a linker. We next attempted to package plasmid DNA (pDNA) in HA-modified carriers by a multifunctional envelope-type nanodevice (MEND) preparation technique. The MEND consists of DNA particles condensed with a polycation and a lipid envelope equipped with various functional devices.[10–14] Furthermore, we report on an investigation of the optimal lipid composition and functional device needed for making strong transgene expression in LECs.

EXPERIMENTAL

Materials

The pDNA encoding enhanced green fluorescent protein and luciferase protein was obtained from BD Bioscience Clontech (Palo Alto, California). pDNA was purified using a Qiagen EndoFree Plasmid Mega Kit (Qiagen GmbH, Hilden, Germany). Egg yolk phosphatidyl choline (EPC) was obtained from Nippon Oil and Fats Company (Tokyo, Japan). Cholesteryl hemisuccinate (CHEMS) and phosphatidic acid (PA) were purchased from Sigma (St. Louis, Mossouri). Cholesterol (Chol), 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 7-nitrobenz-2-oxa-1,3-diazole-labeled DOPE (NBD-DOPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[PEG(2000) maleimide] [DSPE-PEG(2000) maleimide] were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama). Protamine was purchased from Calbio Chem (Darmstadt, Germany). Stearyl octaarginine (STR-R8)[15,16] and cholesteryl GALA peptide[17,18] were obtained from Kurabo Industries Ltd. (Osaka, Japan). HA [average molecular weight (MW) 500–700 kDa, HA (600 kDa)] was obtained from Food Chemifa (Tokyo, Japan). HA preparations with different molecular weights, labeled with fluorescein [average MW 20–30 kDa, HA (25 kDa); average MW 100–300 kDa, HA (200 kDa); average MW 600–1120 kDa, HA (900 kDa)] were purchased from PG Research (Tokyo, Japan). Hoechst 33342 was purchased from Dojindo Laboratories (Kumamoto, Japan). Microvascular Endothelial Cell Growth Medium-2 Bullet Kit (EGM-2 MV) was purchased from Clonetics (Walkersville, Maryland). All other chemicals were commercially available reagent-grade products.

Synthesis of the HA Derivative

A 100-mL, one-necked, round-bottomed flask equipped with a magnetic stirring bar was loaded with 10 mg of HA (600 kDa) (16.6 nmol) in 25 mL of water. 4.79 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) hydrochloride (25.0 μmol) and 2.88 mg of *N*-hydroxysuccinimide (NHS) (25.0 μmol) was added to the reaction mixture, after which 5.63 mg of cystamine dihhydrochloride (25 μmol) was added. The solution was then vigorously stirred overnight at 25°C. The resulting solution was dialyzed using a 5000 molecular weight cut-off dialytic membrane (Spectrum Lab, California) against 1 L of an 80:20 mixture of phosphate-buffered saline (PBS) (−) and methanol and then three times against water, respectively. The solvents were removed with a centrifugal concentrator, and the white solid was collected. The ratio for the incorporation of cystamine was determined by ^{1}H NMR using D_2O as the solvent.

Preparation of HA–PEG–LPs Labeled with NBD

Liposomes labeled with NBD 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 [EPC/Chol/DSPE-PEG(2000) maleimide/NBD-DOPE