Hepatocytes targeting of cationic liposomes modified with soybean sterylglucoside and polyethylene glycol

Xian-Rong Qi, Wen-Wei Yan, Jing Shi

INTRODUCTION
Cationic liposomes have been accepted as effective non-viral vectors for gene delivery with a lower immunogenicity than the viral ones. However, the lack of organ or cell specificity sometimes hampers their applications. In the case of cationic liposomes, the highest gene expression is observed in the lung after intravenous injection of their plasmid DNA complexes in most cases, because the lung capillaries are the first traps to be encountered[18,19]. Development of cell-specific targeting technology for cationic liposomes attracts great interest in gene therapy.

Since the liver is one of most important target organs in the body, and Kupffer cells in the liver are a part of the reticular endothelial system (RES), relatively high accumulation of administered liposomes is observed in the liver, mostly in non-parenchymal cells[3]. However, preferential incorporation of liposomes into liver hepatocytes is required for therapeutic situations. Thus, reducing the Kupffer cell uptake and enhancing hepatocyte uptake, are challenges of research in liposome-targeting.

For cell specific delivery, the receptor-mediated endocytosis systems endowed to various cell types would be useful and a number of gene delivery systems have been developed to introduce gene into specific cells with receptor-mediated endocytosis. Ligands currently being investigated include galactose[6,7], lactosel[8], transferrin[9], etc. Among these receptors, asialoglycoprotein receptor (ASGP-R) is the most promising for gene targeting, since it exhibits high affinity and a rapid internalization rate[10].

It was reported that liposomes with soybean sterylglucoside (SG) gets accumulated in the liver, especially in hepatocytes[9,10]. Doxorubicin (DXR) entrapped in liposomes contained SG (SG-liposomes) showed a high therapeutic effect for its selective delivery of drugs to hepatocytes in animals with liver cancer[11]. SG-liposomes have glucose residue on the surface of liposomes[12], which is essential for selective accumulation in liver cells.

Hepatitis B is a disease of global importance with more than 300 million carriers of the hepatitis B virus (HBV) worldwide[13]. Unfortunately, treatment of chronic HBV infection is far from satisfactory. The most successful therapeutic agent so far available is interferon-alpha, which shows a 40% response rate for patients after completion of the therapy[14,15]. Since several viruses have become successful targets of the ODN approach, this strategy may be promising in targeting chronic HBV infection, and several studies have now shown that ODN are capable of suppressing HBV in vitro[16,17] and in vivo[18,19]. ODN are synthetic single chain DNA molecules that can inhibit gene expression within cells
by their capability to bind a complementary mRNA sequence, and prevent translation of mRNA, thus providing potentially powerful therapeutic tools against viral diseases and cancer. For an ODN delivery system towards HBV infection, the SG may be useful for targeting hepatocytes of the liver.

This study describes a specific targeting approach which results in increased hepatocytes uptake. A cationic liposome carrier modified with SG and encapsulated 15-mer ODN for the HBV therapy in vitro was constructed. The influence of SG on facilitating the uptake of liposomes by hepatocytes was investigated in vitro. The value of the surface modified cationic liposomes as a delivery vehicle, mainly for hepatocytes targeting antisense agent in vitro and in vivo was assessed.

MATERIALS AND METHODS

Synthesis of ODN
ODN with phosphorothioate backbone encoding the cap site of SP II promoter transcribed mRNA (cap site/SP II) sequences were synthesized using standard phosphoramidite chemistry by Aoke (Beijing, China) and purified by SDS-PAGE. The complementary ODN sequences were: 5'-GAT GAC TGT CTC TTA 3'.

Animals and cell line
Male KM mice (18-23 g) were obtained from the Institute of Zoology, Chinese Academy of Sciences (Beijing, China). All animals received good care. A human hepatoblastoma cell line, HepG2.2.15 was provided by the Institute of Hepatology of the People's Hospital, Peking University (Beijing, China).

Materials
N, N-dimethyllethylenediamine (99%) and cholesteryl chloroformate (97%) were obtained from ACROS (USA); dipalmitoylphosphatidylcholine (DPPC) and polyethylene glycol-distearyloxyphosphatidylethanolamine (PEG-DSPPE) were purchased from NOF (Tokyo, Japan); SG was generously supplied by Ryukakusan Co. Ltd. (Tokyo, Japan); cholesterol (Ch) was purchased from Wako Pure Chemical Industries (Tokyo, Japan); 3H-Ch and 125I-ODN was provided by China Institute of Atomic Energy (Beijing, China). FS was obtained from the Third Chemical Reagent Factory of Shanghai (Shanghai, China); DMEM medium and fetal bovine serum (FBS) was purchased from Life Technologies (NY, USA). 2, 5-Diphenyloxazole (PPO) and 1, 4-bis (5-phenyl-2-oxazoyl)-benzene (POPOP) were provided by Fluka (Buchs, Switzerland). Collagenase (II) was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

Synthesis of DC-chol
DC-chol was synthesized according to the method described by Gao. The production was confirmed by thin-layer chromatogram (TLC), melting point, 1H nuclear magnetic resonance (NMR) (500 MHz, CDCl3), mass spectrum (MS), etc.

Preparation of liposomes
The FS or ODN encapsulated liposomes used in the present study were prepared according to the compositions in Table 1, respectively. A mixture of lipids in chloroform was dried under a stream of nitrogen and additionally dried under vacuum for 3 h to remove all chloroform. The dry lipid film was resuspended with FS or ODN solution (solution in 50 g/L glucose) by vortexing and sonicating, and then extruded through 0.2 µm pore size polycarbonate filters to generate the FS and ODN encapsulated liposomes, respectively. The concentration of lipids was 20 µmol/L. To prepare lipid-radioactive labeled liposomes, 3H-Ch was added to the lipid mixture at the beginning of the liposomes preparation and the dry lipid film was resuspended with 5% glucose. The 3H-labeled C-liposomes and C/SG/PEG-liposomes were prepared. The radioactivity of liposomes was 4 µCi/200 µL.

Determination of encapsulation efficacy of liposomes
Free ODN was separated from ODN encapsulated liposomes by equilibrium dialysis, in a dialysis tubing (SpectraPor 12 000 to 14 000 MWCO) at 4 °C for 12 h in 10 mL of 5% glucose solution. The incubation liquid was taken and the concentration of ODN was detected by UV spectro-photometer at 260 nm. Free FS was separated from encapsulated FS by passing through a Sephadex G-50 column (1 cm×20 cm). The concentration of FS was determined by measuring the fluorescence intensity of FS with excitation and emission wavelengths at 490 and 512 nm, respectively. According to the amount of ODN or FS entrapped in the liposomes, the encapsulation efficacy was calculated.

Morphology and size analysis
The size of liposomes was determined by dynamic light scattering using a Zetasizer 3000HS (Malvern Instruments, Ltd., UK). The morphologies of these liposomes were also observed by the transmission electron microscope.

Cell culture and transfection efficiency measurement
HepG2.2.15 was maintained in DMEM medium supplemented with 100 mL/L FBS at 37 °C with 50 mL/L CO2. The cells were scraped by 0.25% trypsin and planted in 96-well tissue culture plates (5×103/well) for 2 d before the experiment, until the percentage of adherent cells reached approximately 70% confluence. The upper medium was removed and fresh DMEM medium was added with 100 mL/L FS encapsulated liposome. When the liposomes were incubated with cells for 3, 6, and 24 h, respectively, the cells were detached with 0.25% trypsin and washed thrice with 10 mmol/L PBS. The transfection efficiency was determined by counting the amount of cells transferred by FS with flow cytometry (BD, USA). The transfection efficiency was calculated according to the following equation: amount of FS transferred cells/amount of total cells×100%. The means of transfection efficiency were calculated from two independent experiments.

Antisense activity of ODN encapsulated in liposomes
For lipofection, HepG2.2.15 cells were seeded at an initial concentration of 1×10⁴ per well for 96-well plates. The cells were allowed to grow for about 24 h, until the percentage
of adherent cells reached approximately 80% confluence. Then the cells were washed extensively to remove the previously secreted HBsAg and HBeAg in the medium. After washing, 100 μL free ODN or C/SG/PEG-liposomes entrapped ODN with an ODN concentration at 1.25, 2.5 or 5.0 μmol/L, together with 100 μL DMEM containing 10% serum, were added. The secretion of HBsAg and HBeAg into the culture supernatants was measured daily for 3 d, using ELISA immunoassay kits. The means of HBsAg and HBeAg immunoassay measurements were calculated from two independent experiments.

Liver uptake in vivo and scintillation counting
Liposomes labeled with 3H-Ch (C-liposomes and C/SG/PEG-liposomes) were injected into the tail vein of three male mice with a dose of 200 μL/20 g. At 0.5 and 4 h after injection, the mice were killed. The liver tissue was collected and washed with saline. About 100 mg of liver samples were decolored in a solution, containing 200 μL HClO4 and 300 μL 60% H2O2. Radiation scintillation fluid was added and mixed thoroughly. The radioactivity (dpm) of samples was counted on a scintillation counter (Pharmacia WALAC 1410, Turku, Finland).

Isolation of liver cells
Mice were injected intravenously by tail vein with 3H-labeled C-liposomes and C/SG/PEG-liposomes, respectively. At 0.5 and 4 h after administration, the mice were anesthetized, and the liver was perfused via the portal vein with isotonic saline to remove the blood. Then the liver was excised, minced and digested in 0.5 g/L collagenase for 30 min at 37 °C. The suspended cells were filtered through cotton mesh sieves, followed by centrifugation at 500 r/min for 3 min. The pellets containing hepatocytes were washed thrice with saline solution by centrifugation at 500 r/min for 3 min. The supernatant containing non-parenchymal cells were similarly centrifuged and washed thrice at 1,500 r/min for 15 min. The radioactivity (dpm) of hepatocytes and non-parenchymal samples was counted on a scintillation counter (Pharmacia WALAC 1410, Turku, Finland).

RESULTS
Characteristics of liposomes
The entrapment efficiencies, size, polydispersity index of all kinds of FS or ODN encapsulated liposomes are shown in Table 1. The results indicated that FS could hardly be

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Enzyme-linked immunosorbent assay (ELISA)
Enzyme-linked immunosorbent assay (ELISA) was used for quantification of HBsAg and HBeAg. The samples were diluted in 10% serum, were added. The secretion of HBsAg and HBeAg into the culture supernatants was measured daily for 3 d, using ELISA immunoassay kits. The means of HBsAg and HBeAg immunoassay measurements were calculated from two independent experiments.

Table 1

| Sample                  | Liposome compositions (molar ratio) | EE (%) | D (nm) | PI  | Entrapment of FS | Entrapment of ODN | E (%) | D (nm) | PI  |
|-------------------------|-----------------------------------|--------|--------|-----|------------------|-------------------|-------|--------|-----|
| N-liposomes             | DPPC/Ch(10:10)                    | 0.64   | -      | -   | -                | -                 | -     | -      | -   |
| C-liposomes             | DPPC/Ch/DC-chol(10:1:10)          | 88.58±4.48\(^1\) | 155.0  | 0.44 | 91.11±5.11\(^1\) | 71.0              | 1.00  |       |     |
| C/SG-liposomes          | DPPC/Ch/DC-chol/SG(10:1:1:1:34)   | 88.46±2.29\(^1\) | 117.8  | 0.31 | -                | 75.4              | 0.44  |       |     |
| C/SG/PEG-liposomes      | DPPC/Ch/DC-chol/PEG/DSPE(10:1:1:3:1:34) | 83.12±3.63\(^3\) | 96.2   | 0.22 | 89.54±1.24\(^2\) | 183.0             | 0.35  |       |     |

\(^1\) Values represent as mean±SD, \(n = 4\). \(^2\) Values represent as mean±SD, \(n = 3\).
and the inhibition effects of C/SG/PEG-liposomes entrapped ODN were increased when incubation time was increased from 24 to 72 h. The inhibition on HBeAg secretion brought by free ODN and C/SG/PEG-liposomes entrapped ODN showed lower tendency compared to the HBsAg. The cells remained viable throughout the experiments and no morphological abnormalities were observed.

**Distribution in liver and intrahepatic cells in vivo**

$^3$H-Ch labeled C-liposomes and C/SG/PEG-liposomes were injected in mice at a dose of 4 $\mu$Ci/20 g. At 0.5 and 4 h after injection, the radioactivity in 100 mg of liver tissue is shown in Figure 3. The distribution amount in liver tissue (total hepatocyte and non-parenchymal cells) was not significantly different for C-liposomes and C/PEG/SG-liposomes. The uptake amounts of liposomes at 0.5 and 4 h in hepatocytes and non-parenchymal cells are also shown in Figure 3. After separating liver cells into hepatocytes and non-parenchymal cells, it was found that the uptake of C/SG/PEG-liposomes was higher than that of C-liposomes by hepatocytes at 0.5 h ($P<0.01$) and the uptake of C/SG/PEG-liposomes was lower than that of C-liposomes by non-parenchymal cells at 0.5 and 4 h ($P<0.01$). These results indicated that the C/SG/PEG-liposomes have more appetency to hepatocytes than non-parenchymal cells in the liver.
DISCUSSION
Among various types of non-viral vector systems, cationic liposomes seem to be promising, because of their high gene expression efficiency. When using simple cationic liposomes by both intravenous and intraportal administration[16,24], it is difficult to transfect into hepatocytes because liposomes prefer targeting the lung and RES. A great challenge faces the investigator who wishes to target liposomes to hepatocytes, for some disorders such as hepatitis B or metabolic diseases which require that the liposomes be steered away from their natural targets. In this study, the characteristics of SG modified liposomes and its inhibition on HBsAg and HBeAg secretion was investigated. In addition, the liver uptake and intrahepatic distribution of a labeled cationic liposome and SG modified cationic liposome were also evaluated.

To investigate the effect of DC-chol, SG and PEG-DSP should be important factors in obtaining a desired inhibition effect. As the ODN and incubation time appeared to be important factors which require that the liposomes be steered away from their natural targets. In this study, the characteristics of SG modified liposomes and its inhibition on HBsAg and HBeAg secretion was investigated. In addition, the liver uptake and intrahepatic distribution of a labeled cationic liposome and SG modified cationic liposome were also evaluated.

To investigate the effect of DC-chol, SG and PEG-DSP on the entrapment and transfection efficiency, FS was used as a marker. DC-chol (positively charged) can bind with FS or ODN (both negatively charged) by electrostatic interaction. Typical cationic liposomes that carry excess positive charge will interact with plasma proteins, and would be rapidly taken up into the mononuclear phagocytic system. In order to decrease the adsorption of plasma proteins and interaction with non-target cells, 6% PEG-DSP was added into the cationic liposomes.

Entrapment of FS and ODN to cationic liposomes was very efficient, even when containing a relatively high amount of SG and PEG-DSP (6%, Table 1). Evidently, SG and PEG coating seldom shields the positively charged liposome surface from interaction with FS and ODN.

Receptors for carbohydrates such as the ASGP-R on hepatocytes and the mannose receptor on macrophages and liver endothelial cells produce opportunities for cell-specific gene delivery with liposomal carriers. The presence of a glucoside on the surface of electrically neutral FS entrapped in C/SG-liposomes, resulted in more than a 2-fold increased transfection efficiency of HepG2 cells, which is a human hepatoblastoma cell line that is known to express ASGP-R, when compared to C-liposomes encapsulated FS (Figure 1). It was surmised that such glucoside in SG could be identified by ASGP-R present on the surface of the HepG2 cells, leading to liposome entry into cells through endocytosis.

The major HBsAg, and in some cases, the HBeAg, is detectable in the serum of individuals with chronically infected HBV. Serological detection of HBeAg usually correlates well with the presence of circulating viremia and is commonly used clinically as an indicator of active HBV replication. Elimination of HBsAg and HBeAg from the supernatant is associated with resolution of infection with a wild-type strain of HBV. Synthetic ODN (15-mers), complementary to the cap site of SP II promoter transcribed mRNA, showed a sequence-specific, dose-dependent inhibitory effect on HBV gene expression from a concentration of 1.25-5.0 μmol/L. A previous study showed that such ODN could inhibit the expression of HBsAg without a significant effect on total synthesized protein in the cells[20]. According to the results of this study, it is found that the initial concentration of ODN and incubation time appeared to be important factors in obtaining a desired inhibition effect. As the ODN concentration and incubation time increased, the inhibition efficiency of C/SG/PEG-liposomes entrapped ODN also increased (Figure 2). However, it is demonstrated that larger amounts of ODN and cationic lipid would lead to a higher toxicity to the cells; therefore, concentration of liposome-ODN complexes should be restricted at a certain level[24]. As for the present ODN encapsulated C/SG/PEG-liposomes, a concentration of DC-chol lower than 10 μg/mL with a cationic lipid/ODN at 1:1 of charge ratio was regarded suitable.

In the study of liver uptake of ³H labeled cationic liposomes, no significant difference was found between C-liposomes and C/SG/PEG-liposomes. These results indicated that the PEG chain and glucose residue in SG did not necessarily improve the in vitro behavior of cationic liposomes. Kirby et al., showed that when the cationic liposomes contain only 5% of charged lipid with small zeta potential, the behavior of the cationic liposomes are not different from that of neutral liposomes[22,23]. Our results agree with these findings. These results could be attributed to the special hepatocytes targeting behavior of SG. A previous study showed that the glucose residue on the surface of liposomes could selectively recognize the ASGP-R of hepatocytes cells[9]. The result also demonstrated that the liver targeting effect of SG is not diminished by the PEG chain on the surface of liposomes.

In conclusion, this study established a highly efficient receptor-mediated delivery system for ODN to hepatocytes by using SG and PEG modified cationic liposomes. By utilizing this delivery system, an ODN targeting the encapsidation site of the HBV pregenome causes a strong inhibition of HBV replication in vitro. Therefore, SG modified liposomes may be effective vehicles to improve the delivery of ODN to the liver for the therapy of hepatotropic viruses.

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