chapter 5

Delivery of viral vectors to hepatic stellate cells in fibrotic livers using HVJ envelopes fused with targeted liposomes

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Abstract
Hepatic stellate cells (HSC) are a major target for antifibrotic therapies in the liver and in particular gene delivery to these cells would be relevant. Previously, we demonstrated that mannose 6-phosphate human serum albumin (M6P-HSA) coupled liposomes accumulate in HSC in fibrotic livers. Here we prepared a M6P-HSA modified viral vector that allows the targeted delivery of plasmid DNA to HSC. Therefore, UV inactivated Hemagglutinating virus of Japan (HVJ) containing plasmid DNA was fused with M6P-HSA liposomes to yield HVJ liposomes targeted to HSC. These new particles had a diameter of approximately 200 nm, as determined by electron microscopy. In a carbon tetrachloride mouse model of liver fibrosis, M6P-HSA-HVJ-liposomes associated with HSC. In conclusion, our results demonstrate that fusion of M6P-HSA liposomes with HVJ envelopes results in HVJ particles that accumulate in HSC, allowing new possibilities to interfere with fibrosis in the liver.
Introduction

Upon liver injury, Kupffer cells, hepatocytes and sinusoidal endothelial cells are stimulated so as to effectuate the transformation of quiescent HSC into an activated phenotype that is characterised by a high rate of extracellular matrix production and its deposition in the organ. In addition, activated HSC proliferate and migrate towards areas of injury and produce chemokines which attract immune cells from the circulation (1). These actions of HSC cause a perpetuation of the fibrotic process in the liver, eventually leading to cirrhosis. As a result, HSC are considered a major target for therapies against liver fibrosis. A variety of strategies is currently being tested for their antifibrotic potential and among others therapeutic gene modulation is considered as a viable approach towards treatment of fibrosis (2). Viral gene delivery systems might be an alternative to conventional treatment. It has already been demonstrated that delivery of genes encoding proteins, like interferon α (3), superoxide dismutase (4), matrix metalloproteinase-8 (5) and Smad7 (6) as well as antisense mRNA to transforming growth factor-β 1 (7) using adenoviral vectors can attenuate liver fibrosis in rat models of the disease. Because adenoviruses specifically interact with hepatocytes, the vectors used in these studies mostly transfected hepatocytes while delivery them to other cells is difficult.

The hemagglutinating virus of Japan (HVJ), also known as Sendai virus, has an average diameter of 200 – 400 nm. Its lipid membrane contains two glycoproteins: hemagglutinin-neuraminidase (HN) and the fusion protein F. Both proteins are essential for fusion of the virus with the plasma membrane of the host cells, the process by which HVJ infect cells. HN binds to sialoglycoproteins on the surface of the cell membrane. The actual fusion occurs after exposure of a hydrophobic region of the F protein and its interaction with the lipid bilayer of the cell membrane (8).

Earlier we demonstrated that HVJ is able to fuse with conventional liposomes, even though they do not contain sialoglycoproteins on their surface (9). During this fusion process, membrane proteins of HVJ are incorporated into the liposomal membrane. Several reports showed that vectors derived from the fusion of liposomes that contain plasmid DNA or oligonucleotides with inactivated HVJ form an effective gene transfection system, both in vitro and in vivo (10-13). However, these HVJ-liposomes lack tissue or cell selectivity. Previously we demonstrated that liposomes that are surface coupled with mannose 6-phosphate modified human serum albumin (M6P-HSA) accumulate in HSC in a rat model of liver fibrosis (14). The combine HSC specific targeting with the effective HVJ gene transfection system may offer the possibility to construct a new vector, characterised by the delivery of therapeutic genes selectively to HSC and an enhanced transfection efficiency of these pivotal cells during fibrogenesis.

In the present study, we therefore tested if fusion can be accomplished between M6P-HSA liposomes and HVJ envelopes containing plasmid DNA (15) and if the HVJ particles thus obtained can be targeted to HSC. The new particles resulting from the fusion process were characterised with regard their size and their phospholipid, protein and DNA content. The transfection efficiency of the targeted HVJ particles was tested in vitro and selective targeting to HSC was assessed in a mouse model of liver fibrosis.

Materials and Methods
Chemicals
Cholesterol (Chol), egg 1,2-diacetyl-sn-glycero-3-phosphocholine (PC), 1,2-dioleoyl-sn-
glycero-3-phosphoethanolamine (DOPE), N-succinimidyl-S-acetylthioacetate (SATA) were obtained from Sigma (St. Louis, MO, USA). 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Maleimide(Polyethylene Glycol)2000] (DSPE-PEG2000-MAL) was purchased from Avanti Polar Lipids (Alabaster AL, USA). Human serum albumin fraction V came from the Sanquin blood bank (Amsterdam, The Netherlands). Luciferase expression plasmid driven by the cytomegalovirus promoter was purchased from Promega (Madison, WI, USA). All other chemicals were of analytical grade or the best grade available.

Preparation of HVJ envelope and incorporation of plasmid DNA
The Z strain of Hemagglutinating virus of Japan (HVJ) was propagated in chick eggs and purified by centrifugation as described before (16). HVJ-E was prepared as reported before (16). Shortly, HVJ (10 000 Hemagglutinating units, HAU) was inactivated by UV irradiation (99 mJ/cm²), centrifuged for 10 min at 15 000 rpm. The pellet was resuspend in solution of protamine sulphate (1 mg/ml), plasmid DNA (200 μg) containing luciferase as a reporter gene (pDNA-Luc) and a solution containing 3 % Triton X-100 was added and the mixture was centrifuged for 15 min at 15 000 rpm, 4°C. To remove unincorporated DNA and detergent the pellet was washed with balanced salt solution (10 mM Tris-Cl, pH 7.5, 137 mM NaCl and 5.4 mM KCl). The obtained HVJ envelope was resuspended in PBS and stored at 4°C until use.

Preparation of M6P-HSA
Synthesis of human serum albumin modified with mannose 6-phosphate groups was performed as described before (17). In order to couple M6P-HSA to liposomes, active SH groups were introduced to the protein using the SATA method (18). M6P-HSA used in this study contained three SH groups per molecule. SATA modified M6P-HSA was stored at -20°C until use.

Preparation of liposomes
Liposomes composed of PC/CHOL/DOPE/DSPE-PEG2000-MAL in the molar ratio 21.5:16:2:0.5 were prepared as reported before (19). Briefly, lipids from chloroform: methanol (9:1) stock solutions were pipetted in a glass tube at the indicated molar ratio and organic solvent was evaporated under nitrogen. Dried lipids were dissolved in cyclohexane and freeze-dried over night. The dried lipids were hydrated with HNE (10 mM HEPES, 135 mM NaCl, 0.1 mM EDTA) buffer pH 6.7 and the liposomes thus formed were sized by repeated extrusion through polycarbonate filters (50 nm). The concentration of each liposome preparation was determined by measurement of phospholipid phosphorus (20). The total lipid concentration was calculated taking into account the amount of cholesterol in the liposomal lipid mixture. M6P-HSA was coupled to the liposomes as described before (21). Briefly, SATA-modified M6P-HSA was incubated with the liposomes for 4 h at room temperature (0.3 mg protein / μmol of liposomal lipid). The coupling reaction was stopped by addition of 10 mM N-ethylmaleimide (Sigma) and uncoupled M6P-HSA was removed by ultracentrifugation (2
x 2h at 40,000 rpm, 4°C) in Opti-Prep™ (Axis-Shield PoCAS, Oslo, Norway). The amount of M6P-HSA coupled to the liposomes was measured by DC Protein assay (Bio-Rad).

**Fusion of HVJ envelope with M6P-HSA liposomes**

HVJ envelopes containing pDNA-Luc were incubated with M6P-HSA liposomes (15,000 HAU per 15 μmol total lipids of M6P-HSA liposomes) on ice for 10 min and subsequently, 1 h at 37°C in a shaking water bath. Fused liposomes were separated from free HVJ and unfused liposomes by discontinuous sucrose density gradient centrifugation (16). From bottom to top the centrifuge tube contained 1 ml 60% sucrose solution followed by 7 ml 30% sucrose and 1.5 ml incubation mixture. The tubes were centrifuged for 90 min at 20,000 rpm, 4°C and the fractions obtained after centrifugation were collected and dialysed overnight against HNE buffer pH 7.4.

**Characterisation of fused M6P-HSA-HVJ liposomes**

**Quantitative measurements**

To determine the concentration of fused M6P-HSA-HVJ liposomes, and the overall ratio in which fusion had taken place, independent measurements were done of the protein and phosphate concentrations of M6P-HSA liposomes and HVJ envelopes. Using these, a system of linear equations could be constructed for the M6P-HSA-HVJ liposomes, which was solved to find the separate contributions of these components from measurements on the fused particles. Generally, if \( p_l \) and \( p_e \) are the protein concentrations for M6P-HSA liposomes and HVJ envelopes, respectively, and \( l_l \) and \( l_e \) the lipid concentrations for both, the concentration of liposomes \( C_l \) (μmol Pi) and HVJ envelopes \( C_e \) (HAU) are given by

\[
C_l = \frac{p_l l_l - L p_l}{l_l p_e - p_l l_e}, \quad C_e = \frac{L p_e - p_l l_e}{l_l p_e - p_l l_e},
\]

with \( P \) and \( L \) the measured concentrations of protein and lipid in the fused particles, respectively.

**Electron microscopy**

Electron microscopic examination of the samples was performed after negative staining with a 1.5% solution of phosphotungstic acid, using Hitachi TEM system (Hitachi, Ibaragi, Japan). The size of the particles was estimated by comparison to scale bar, generated by the TEM system.

**SDS-PAGE analysis**

The proteins of the HVJ envelopes, the M6P-HSA-HVJ liposomes and M6P-HSA (all 2 μg protein/lane) were separated on 10% SDS-PAGE and stained with a Silver Stain kit (Bio-Rad, CA, USA) according to the protocol of manufacturer.

**Agarose gel electrophoresis**

In order to confirm presence of the plasmid DNA in M6P-HSA-HVJ liposomes, these particles (50 HAU) were analysed on a 1% agarose gel.
Luciferase gene expression assay

HEK 293 cells (human embryonic kidney cells) were cultured in Minimum Essential Medium (Gibco-BRL, Rockville, MD, USA) containing 10% foetal calf serum (Bio West, Miami, FL, USA) and penicillin, streptomycin (Nakarai Tesque, Kyoto, Japan). A day before the experiment cells were seeded at a density of 50,000 cells/well in 24 wells plates (Costar) and cultured overnight. Subsequently, cells were incubated with M6P-HSA-HVJ liposomes or HVJ-E at a concentration of 250 HAU for 4 h, after which the incubation medium was removed and the incubation was continued with fresh medium for another 48 h. Incubations were stopped by washing the cells with PBS and cells were lysed with Passive Lysis Buffer (Promega). The luciferase activity in the cell lysates was measured using a Luciferase Assay kit (Promega).

In vivo studies in mice with experimental liver fibrosis

Targeting of M6P-HSA-HVJ liposomes was tested in a mouse CCl4 model of liver fibrosis. C57 BL/6 mice were injected (i.p) twice a week for 4 weeks with CCl4 (1ml/kg of body weight), diluted in corn oil (1:10) (22,23). Three days after the last injection, the mice were injected via the tail vein with M6P-HSA-HVJ liposomes (1000 HAU) or HVJ-E (1000 HAU). One hour after injection of the particles the mice were sacrificed and the livers were removed for immunohistochemical analysis.

Immunohistochemical analysis

To detect M6P receptors, liver sections were fixed in acetone and incubated with a goat antibody directed against M6P receptor (Santa Cruz Biotechnology, Inc). Subsequently, endogenous peroxidase was inhibited with H2O2, followed by incubation with peroxidase conjugated rabbit-anti-goat IgG (RaGPo, Dako) and amplification with peroxidase conjugated goat-anti-rabbit IgG (GaRPo, Dako). Antibody associated peroxidase was visualised with 3-amino-9-ethyl-carbazole (AEC).

To detect HVJ in the liver, sections were fixed with 4% paraformaldehyde using the Vector® M.O.M. Immunodetection kit (Vector Laboratories, Inc, CA, USA) according to the protocol of the manufacturer with minor modifications. Briefly, sections were blocked with M.O.M™ Mouse Ig Blocking Reagent and incubated with a mouse monoclonal antibody against F protein, diluted 1:50 in M.O.M™ Diluent. Subsequently, Alexa-488 conjugated goat anti-mouse antibody (Molecular Probes, Oregon, USA) diluted in M.O.M™ Diluent was used as a secondary antibody and the nuclei of the cells were stained with DAPI. Staining was analysed by fluorescence microscopy and confocal laser scanning microscopy.

To analyse co-localisation of M6P-HSA-HVJ liposomes with HSC liver sections were fixed in acetone and the immunodetection was performed using the Vector® M.O.M. Immunodetection kit according to the protocol of the manufacturer with minor modifications. Sections were blocked with M.O.M™ Mouse Ig Blocking Reagent. Desmin, a HSC marker was detected with an anti-desmin monoclonal antibody (Sigma) followed by a second step with rabbit anti-mouse FITC conjugated antibody (Dako Cytomation, Denmark). HSA was stained with rabbit anti-HSA antibody (Cappel) followed by swine anti-rabbit TRIC conjugated antibody (Dako). All used antibodies were diluted in M.O.M™ Diluent. Stained sections were analysed using a Leica fluorescence microscope.
Results

Quantitative analysis of particles after fusion of HVJ-E and M6P-HSA liposomes

HVJ liposomes containing the luciferase gene were fused with M6P-HSA liposomes and the mixture obtained was centrifuged on a discontinuous sucrose density gradient. Thus, three fractions were obtained (Fig. 1A), one (fraction I) near the top of the gradient, another one (fraction II) at the interface between the 30% sucrose and the buffer and the heaviest third one (fraction III) at the interface between 30% and 60% sucrose. The three fractions were quantitatively analysed for phosphorus and protein content and from these values we calculated the relative contribution of the HVJ-E (hemagglutinating units, HAU) and M6P-HSA liposomes (phosphate, Pi) in each fraction, as described in the Materials and Methods section. Fraction III contained 50% of HVJ-E applied to the gradient but did not contain significant concentrations of Pi, indicative of the absence of HVJ-E and M6P-HSA liposome fusion product in this fraction (Table 1). By contrast, fraction I contained 30% of the HVJ-E derived material and around 70% of the initial M6P-HSA liposome-derived material. In the fraction II only low amounts of HVJ-E and liposome-driven material were detected.

Qualitative analysis of particles after fusion of HVJ-E and M6P-HSA liposomes

Table 1. Quantitative analysis of particles after fusion of HVJ-E and M6P-HSA liposomes.

<table>
<thead>
<tr>
<th>fraction</th>
<th>% of HVJ-E</th>
<th>% of M6P-HSA-L</th>
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<tbody>
<tr>
<td>fraction I</td>
<td>31 ± 9</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>fraction II</td>
<td>12 ± 2</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>fraction III</td>
<td>53 ± 2</td>
<td>6 ± 5</td>
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HVJ-E were mixed with M6P-HSA liposomes in a ratio of 1000 HAU of HVJ-E per μmol total lipid of liposomes and after incubation the mixture was centrifuged on a sucrose density gradient, as described in Material and Methods. Three fractions were collected from the gradient as described in Fig. 1A and analysed for the present quantity of HVJ-E material, expressed as % of total added HVJ-E, and of M6P-HSA liposome-derived material, expressed as % of added liposomes. Data are presented as mean ± SEM of 3 preparations.

In order to identify the proteins present in each fraction collected from the sucrose density gradients, they were analysed by polyacrylamide gel electrophoresis. As presented in Fig. 1B, fraction III contained the same types of proteins as HVJ-E but hardly any M6P-HSA. In fraction I we found all HVJ-E proteins in addition to a strong band of M6P-HSA. While the appearance of the second fraction on the gel was similar to the first fraction. Electron microscopy revealed that fraction I consisted of a rather homogenous population of particles with a size was of around 100 nm (Fig 2 A). Particles in the second fraction were 50-200 nm. In addition, this fraction also contained apparently destroyed particles and debris. The particles in fraction III varied in size from 200 to 400 nm and were identified as HVJ envelopes.
Luciferase gene expression assay

The transfection potential of the particles from the sucrose density gradient was tested on HEK 293 cells using a luciferase gene expression assay. We observed that after 48 h the expression of luciferase in the cells incubated with particles from fraction III was high and almost equal to that in the cells transfected with unmodified HVJ-E alone (Fig. 3A). Significant expression of luciferase activity was also observed in cells transfected with fraction II. However, the cells incubated with the particles of fraction I, which are most
likely to represent real fusion product of envelopes and liposomes did not show significant luciferase expression. The three gradient fractions were also assayed for pDNA-Luc content by means of agarose gel electrophoresis (Fig. 3B). As expected on the basis of its low transfection activity, fraction I contained very little DNA.

Targeting of fused particles to hepatic stellate cells in the fibrotic liver

To test whether particles formed during fusion of HVJ-E and M6P-HSA liposomes are able to target HSC, we performed in vivo studies in a CCl₄ mouse model of liver fibrosis. In the livers of these mice, substantial M6P receptor expression was observed in both hepatocytes and non-parenchymal cells (Fig. 4).

We compared the targeting properties of fraction I particles and of HVJ-E by means of immunohistochemical analysis. Immunostaining with an antibody against the F protein of HVJ showed that fraction I particles as well as HVJ-E were abundantly present in the livers as early as 1 h after injection, but with remarkably different sinusoidal distribution patterns (Fig. 5). HVJ-E were detected as bright individual spots (Fig. 5A) while in mice injected with M6P-HSA-HVJ-liposomes the F protein was detected in diffuse elongated shapes, presumably representing the sinusoidal lining (Fig. 5B). For both signals confocal microscopy confirmed that they were cell associated (Fig. 5 C, D). In the livers of mice injected with fraction I particles we were also able to detect separately the liposomes-derived part of the particles by means of immunostaining for HSA (Fig. 5 E). Finally, simultaneous detection of the HSC marker desmin and HSA in the livers of mice injected with fraction I, revealed that the two signals were localised in close proximity indicating that the HSA was localised within HSC (Fig. 5 F).
Figure 4. Expression of M6P receptors in fibrotic livers of CCl₄-treated mouse. M6P receptor was detected in control mice injected with only corn oil (A) and in CCl₄ treated mice (B) using immunohistochemical analysis as described in Materials and Methods. Original magnification x 400.

Figure 5. Targeting of fused HVJ liposomes to HSC in fibrotic livers. M6P-HSA-HVJ-liposomes (fraction I) or HVJ-E were injected into mice at a dose of 1000 HAU. In tissue sections of livers obtained 1 h after injection, particles were detected using an antibody against F protein (A, C) HVJ-E, (B, D) M6P-HSA-HVJ-liposomes, magnification x 200. (C) and (D) show representative pictures obtained after examination of sections with a confocal microscope. Note the co-localisation of green fluorescent signal with the nuclei of the cells (arrows). (E) and (F) show representative photographs after immunostaining for the HSA (E, red signal, x 200) and after simultaneous detection of HSA (red staining) and the HSC marker, desmin (F, green signal, x 400). Note the co-localisation of HSA with desmin (arrows). Nuclei of the cells were stained with DAPI (blue).
Discussion

We demonstrated that fusion of HVJ envelopes containing plasmid DNA with M6P-HSA liposomes results in the formation of new particles, M6P-HSA-HVJ-liposomes. These particles were collected as the top fraction from a sucrose density gradient and formed a rather homogeneous population with an average size of around 100 nm. As we reported before, M6P-HSA coupled to the surface of liposomes can target these to HSC in fibrotic livers (14). HSC are situated in the space of Disse, between hepatocytes and endothelial cells, in the close contact with the latter. As a result, blood-borne substances can only reach these cells after crossing the 150 - 200 nm fenestrations in the endothelial cells (24). The size of the M6P-HSA-HVJ fusion product particles allows them to pass the fenestrations in the endothelial lining and thus to be taken up by HSC. Indeed, in the mouse CCl4 model of liver fibrosis, we showed that M6P-HSA-HVJ-liposomes are able to pass the fenestrations and home to the HSC. In contrast, HVJ-E which do not contain M6P-HSA and have a size of over 200 nm, seem to be taken up mainly by Kupffer cells as indicated by the bright punctuate appearance in liver sections tissue. Although M6P receptors expression was observed on hepatocytes as well, we did not observe accumulation of the M6P-HSA-HVJ-liposomes in these cells. In hepatocytes the M6P-binding receptor are present within the cells where they play a role in the shuffling of proteins to lysosomes. These receptors are recognised by antibodies on the fixed liver sections but M6P-containing liposomes can not bind extracellularly to them.

The small size of the M6P-HSA-HVJ-liposomes (fraction I) might explain the low transfection efficiency we found in vitro. Analysis of the plasmid DNA content showed that this fraction contained considerably less plasmid DNA than the remaining unfused HVJ-E (fraction III). Smaller size siRNA or antisense oligonucleotides might be incorporated more efficiently in M6P-HSA-HVJ-liposomes than plasmid DNA. HVJ-E have been shown efficient in functional delivery of antisense oligonucleotides (11;25). An alternative explanation of the lack of transfection activity of fraction I may be that the insertion of the M6P-HSA in the bilayer of the viral envelopes interferes with the natural route by which the native virus enters cells and may, as a consequence, lower the transfection efficiency of the modified HVJ-E. The hemagglutinin-neuraminidase (HN) of the native virus binds to sialoglycoproteins on the cell surface. Subsequently, a hydrophobic region of the F protein is exposed, perturbing the cell membrane and allowing fusion between virus and cell and delivery of the nucleocapsid in the cytoplasm thus circumventing the lysosomal compartment (8). The presence of M6P-HSA on the surface of the modified HVJ-liposomes may direct the particles to enter the cells via M6P receptors followed by delivery into the lysosomal pathway which is the natural route for ligands of these receptors, once inside the cell (26). Although, despite the uptake by receptor-mediated endocytosis, part of the M6P-HSA-HVJ-liposomes and/or its DNA cargo might still escape the endosomes and gain access to the cytoplasm, the bulk of these particles is likely to be degraded in the lysosomes. Further studies will be required to elucidate the mechanism of the intracellular routing of M6P-HSA-HVJ-liposomes.

As a result of tissue damage in fibrotic livers, hepatocytes release reactive oxygen species and cytokines which activate Kupffer cells. The growth factors and cytokines that are subsequently produced by the Kupffer cells activate in turn the HSC, which than differentiate into myofibroblast-like cells. At this stage, the HSC do not only produce large amounts of extracellular matrix proteins, but they also secrete transforming growth factor
β (TGF-β) and compounds such as free radicals, monocyte chemoattractant protein–1 (MCP-1) and endothelin–1, which in an autocrine manner perpetuate the activated state of HSC and hence the progression of fibrosis (27). Selective intervention aimed at bringing the activation of HSC to halt and preventing the subsequent extracellular matrix production by these cells is considered to be crucial for improvement of current antifibrotic therapies. The delivery to HSC of either specific genes or siRNA, which at the gene expression level could for example inhibit collagen production or interfere with the signalling pathways of TGF-β or platelet derived growth factor (PDGF), may provide means to design new strategies in the treatment of liver fibrosis.

In conclusion, we demonstrated that the fusion of HVJ-E with M6P-HSA liposomes creates new targeted particles, which in an experimental model of liver fibrosis were shown to accumulate in HSC. Since this cell type plays a crucial role in the development of liver fibrosis, these novel M6P-HSA-HVJ-liposomes may provide new opportunities to modulate the expression of essential genes during the development of liver fibrosis.

References


25. Tomita N, Morishita R, Yamamoto K,


The high pressure extruder — ‘beloved’ piece of equipment. A total of 82 preparations of liposomes were done over a period of 4 years.