Effects of a New Bioactive Lipid-Based Drug Carrier on Cultured Hepatic Stellate Cells and Liver Fibrosis in Bile Duct-Ligated Rats

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ABSTRACT

In the fibrotic liver, hepatic stellate cells (HSC) produce large amounts of collagen and secrete variety of mediators that promote development of fibrosis in this organ. Therefore, these cells are considered an attractive target for antifibrotic therapies. We incorporated the bioactive lipid dilinoleoylphosphatidylcholine (DLPC) into the membrane of liposomes, and then we evaluated its effect on hepatic stellate cell activation and liver fibrosis. To target DLPC-liposomes to HSC, human serum albumin modified with mannose 6-phosphate (M6P-HSA) was coupled to the surface of these liposomes. In vitro, the effects of the carrier were determined in primary cultures of HSC, Kupffer cells, and liver endothelial cells using real-time reverse transcription-polymerase chain reaction. In vivo, DLPC-liposomes were tested in bile duct-ligated rats. Targeted M6P-HSA-DLPC-liposomes significantly reduced gene expression levels for collagen 1α1, α-smooth muscle actin (α-SMA), and transforming growth factor-β (TGF-β) in cultured HSC. In fibrotic livers, DLPC-liposomes decreased gene expression for TGF-β and collagen 1α1 as well as α-SMA and collagen protein expression. In contrast, M6P-HSA-DLPC-liposomes enhanced expression of profibrotic and proinflammatory genes in vivo. In cultured Kupffer and endothelial cells M6P-HSA liposomes influenced the expression of proinflammatory genes. Both types of liposomes increased hepatocyte glycogen content in fibrotic livers, indicating improved functionality of the hepatocytes. We conclude that DLPC-containing liposomes attenuate activation of cultured HSC. In fibrotic livers, M6P-HSA-mediated activation of Kupffer and endothelial cells probably counteracts this beneficial effect of DLPC-liposomes. Therefore, these bioactive drug carriers modulate the activity of all liver cells during liver fibrosis.

Chronic liver injury may lead to development of fibrosis, a process in which hepatic stellate cells (HSC) play a major role. As a result of liver injury, HSC, which in the healthy organ store vitamin A, undergo a process of activation that is mediated by the concerted action of resident hepatic cell types such as Kupffer cells (KC), liver endothelial cells (LEC), and hepatocytes. The phenotype of activated HSC resembles that of myofibroblasts and is characterized by α-smooth muscle actin (α-SMA) expression; intensive synthesis of extracellular matrix proteins, mainly collagen type I and type III; and a high rate of proliferation (Friedman, 2003; Bataller and Brenner, 2005). In addition to this, activated HSC secrete profibrotic and proinflammatory mediators, which, in an autocrine manner, perpetuate the activated state of HSC and attract immune cells from the bloodstream.

ABBREVIATIONS: HSC, hepatic stellate cell(s); KC, Kupffer cell(s); LEC, liver endothelial cell(s); α-SMA, α-smooth muscle actin; M6P, mannose 6-phosphate; HSA, human serum albumin; IGF, insulin-like growth factor; DLPC, dilinoleoylphosphatidylcholine; TNF, tumor necrosis factor; CHOL, cholesterol; SATA, N-succinimidyl-3-acetylthioacetate; POPC, palmitoyleoylphosphatidylcholine; MPB-PE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(maleimidophenyl)butyramide]; FCS, fetal calf serum; d-3, day 3; d-10, day 10; PBS, phosphate-buffered saline; BDL, bile duct ligation/ligated; RT-PCR, reverse transcription-polymerase chain reaction; TGF, transforming growth factor; IL, interleukin; MCP, monocyte chemoattractant protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cq, threshold cycle; PUFAs, polyunsaturated fatty acids(s); LA, linoleic acid; L, liposomes; PAS, periodic acid-Schiff staining.
(Maher, 2001; Pinzani and Marra, 2001). In addition, the contractile features of activated HSC are the basis for their pivotal role in the portal hypertension, which is a major clinical characteristic of this disease (Reynaert et al., 2002).

Any antifibrotic therapy that would uniquely and specifically address the HSC population would signify a major breakthrough in the therapeutic treatment of this disease. Although current antifibrotic drugs that aim at HSC often show promising effects in vitro, their effectiveness is rather limited when tested in vivo. A strategy to enhance the delivery of such drugs to the relevant cells within the liver can be expected to lead to considerable improvement of the success of such treatments. Liposomes, biodegradable lipid nanospheres that can encapsulate drugs with high efficiency, are considered to represent a suitable and versatile drug carrier system. Recently, we showed efficient targeted delivery of liposomes to HSC in the fibrotic liver by coupling human serum albumin modified with mannose 6-phosphate groups (M6P-HSA) to the liposomal surface (Adrian et al., 2006). We also demonstrated that in the interaction of these liposomes with the cells mannose 6-phosphate/insulin-like growth factor II (M6P/IGF II) receptor and scavenger receptor are involved.

It has been reported that dilinoleoylphosphatidylcholine (DLPC) has beneficial effects on cultured HSC. DLPC reduces the activation and proliferation of HSC (Poniachik et al., 1999; Cao et al., 2002c,d). In addition, DLPC decreases tumor necrosis factor (TNF)-α production by Kupffer cells (Cao et al., 2002a,b).

Liposomes with DLPC as a major constituent might therefore function as a bioactive drug carrier that can deliver drugs and simultaneously have beneficial antifibrotic effects. In the present study, we incorporated DLPC into the lipid bilayer of liposomes. To target them to HSC, the surface of DLPC-containing liposomes was modified with M6P-HSA. We evaluated the antifibrotic properties of this new drug carrier on cultured HSC, by testing its effects on the expression of profibrotic genes. In addition, we investigated whether DLPC-containing liposomes can modulate gene expression in cultured KC and LEC. The influence of DLPC-containing liposomes on the fibrotic process in the liver was evaluated in a bile duct ligation rat model of liver fibrosis. We provide evidence that DLPC-containing liposomes are endowed with antifibrotic properties and that they may, while serving as carriers for other antifibrotic compounds, be themselves bioactive.

Materials and Methods

Chemicals

Cholesterol (CHOL) and N-succinimidyl-S-acetylthioacetate (SATA) were from Sigma-Aldrich (St. Louis, MO). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), DLPC, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(maleimidophenyl)butyramide] (MPB-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). The chemical structures of lipids used in this report can be found on http://www.avantilipids.com. Human serum albumin fraction V was from Sanquin (Amsterdam, The Netherlands). Dulbecco’s modified Eagle’s medium, RPMI 1640 medium, and L-glutamine were obtained from Invitrogen (Paisley, Scotland). Fetal calf serum (FCS) was from Cambrex Bio Science (Verviers, Belgium). Penicillin and streptomycin from Sigma-Aldrich. All other chemicals were of analytical grade or the best grade available.

Animals

Specified pathogen free male Wistar and Wag/Rij rats (Harlan, Horst, The Netherlands) were housed under standard laboratory conditions with free access to food and water. The presented studies were approved by the local committee for care and use of laboratory animals.

Preparation of M6P-HSA

Synthesis of human serum albumin modified with mannose 6-phosphate groups was performed as described previously (Beljaars et al., 1999).

Preparation of Liposomes

Liposomes composed of either POPC/CHOL/MPB-PE or DLPC/CHOL/MPB-PE in a molar ratio 23:16:1 were prepared as reported previously (Kamps et al., 1999). In brief, the lipids were taken from chloroform/methanol (9:1) stock solutions, the organic solvent was evaporated under nitrogen, cyclohexane was added, and the lipid solution was lyophilized overnight. The dried powdery lipids were hydrated in HN buffer (10 mM HEPES and 135 mM NaCl, pH 6.7) for 1 h at room temperature. Liposomes were sized by extrusion through polycarbonate filters Whatman (Maidston, Kent, UK) of 50-nm pore size using a high-pressure extruder (Lipex Biomembranes, Vancouver, BC, Canada). The size of liposomes was measured using dynamic laser light scattering with a submicron particle analyzer (NICOMP 380 ZLS; Nicomp, Santa Barbara, CA). The diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. The lipid concentration of each preparation of liposomes was determined by phosphate assay (Böttcher et al., 1961). The total lipid concentration was calculated taking into account the amount of cholesterol in the liposomal preparation.

Coupling of M6P-HSA to liposomes was performed according to the method described previously (Kamps et al., 1996). In brief, sulfydryl groups were introduced into the M6P-HSA using SATA reagent. Liposomes were incubated with SATA-modified protein for 4 h at room temperature; the coupling reaction was stopped by adding 80 mM N-ethylmaleimide (Sigma-Aldrich). Uncoupled M6P-HSA was removed by ultracentrifugation (2 × 2 h at 40,000 rpm at 4°C) in Opti-Prep (Axis-Shield PoCAS, Oslo, Norway) and dialyzed overnight against HN buffer, pH 7.4. After coupling M6P-HSA, liposomes were again characterized by size measurement and lipid concentration. The amount of coupled protein was determined according to Peterson-Loewy (Peterson, 1977). Liposomes were stored at 4°C under argon, and they were used for experiments within 3 weeks after preparation.

Isolation and Culture of Rat HSC, KC, and LEC

HSC were isolated from livers of male Wistar rats (550–600 g) as described previously (Geerts et al., 1998). Isolated HSC were cultured in Dulbecco’s modified Eagle’s medium containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. For experiments performed 3 days after HSC isolation (quiescent phenotype, HSC d-3), cells were seeded on plates directly after isolation as indicated below. For experiments performed 10 days after isolation (activated phenotype, HSC d-10), cells were seeded on plates 3 days after the start of the experiments, the cells were trypsinized and seeded in plates as indicated below.

KC and LEC were isolated from livers of male Wag/Rij rats (200–250 g) (Harlan) as described previously (Kamps et al., 1999). KC and LEC were cultured in RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and in the case of LEC with 10 ng/ml endothelial cell growth factor (Roche Diagnostics, Mannheim, Germany). KC and LEC (initially 350,000 cells/cm²) were cultured for 2 days on 12-wells plates (Corning Life Sciences) and 24-well collagen-coated plates (Greiner...
Bio-One GmbH, Frickenhausen, Germany), respectively, before being used in the experiments.

**Effects of DLPC-Containing Liposomes on Cultured Cells**

Effects of DLPC-Containing Liposomes or Linoleic Acid on Gene Expression Levels in Cultured Cells. HSC were seeded on 12-wells plates (HSC d-3, 200,000 cells/well and HSC d-10, 125,000 cells/well). KC and LEC were plated as described in the preceding paragraph. On the day of the experiment, cells were washed with culture medium without FCS, and they were subsequently incubated for 24 h with culture medium containing 5% FCS supplemented with liposomes at a concentration of 320 nmol lipid/ml or 30 μg/ml M6P-HSA or with 0.1 mM linoleic acid (Sigma-Aldrich) diluted in ethanol. The incubation was stopped by washing the cells twice with PBS, and cells were lysed with lysis buffer provided with the Absolutely RNA MicroPrep kit (Stratagene, Amsterdam, The Netherlands). Collected samples were stored at −80°C until further analysis.

Viability Assay. HSC were seeded in 96-well plates (HSC d-3, 10,000 cells/well and HSC d-10, 5000 cells/well). On the day of the experiment, cells were washed with culture medium without FCS, and liposomes (final concentration 320 nmol lipids/ml) or 30 μg/ml M6P-HSA were added to the culture medium containing 5% FCS. After 4 h of incubation, AlamarBlue reagent (Serotec, Oxford, UK) was added (10% of the total volume), and the incubation was continued for another 20 h. Subsequently, the incubation mixture was transferred into a white 96-well plate (Corning Life Sciences), and fluorescence signal was measured on PerkinElmer LS 50B (PerkinElmer Ltd., Beaconsfield, Buckinghamshire, England) with excitation wavelength at 560 nm and emission wavelength at 590 nm.

Effects of DLPC-Containing Liposomes on Liver Fibrosis in Bile Duct-Ligated Rats

To induce liver fibrosis, bile duct ligation (BDL) was performed in male Wistar rats (250 g) under anesthesia with O2/N2O/Forene (isoflurane; Abbott Laboratories Ltd., Queensborough, UK) according to standard procedures (Kountouras et al., 1984). Ten days after bile duct ligation, rats were treated with M6P-HSA DLPC-liposomes, DLPC-liposomes, M6P-HSA POPC-liposomes, or POPC-liposomes at a dose of 3 μmol of lipid per 100 g of body weight. Control animals were injected with PBS. After 24 h, rats were killed, and samples of the livers were snap frozen in isopentane and stored at −80°C for further immunohistochemical and real-time RT-PCR analysis.

Histological Analysis

Immunohistochemical Staining. Sections of the livers (4 μm) were fixed with acetone, rehydrated in PBS, and incubated either with primary mouse antibodies directed against α-smooth muscle actin (Sigma-Aldrich) or with primary goat antibodies directed against collagen type I and type III (Biological Technology Associates, Birmingham, AL). Endogenous peroxidase activity was inhibited by a 0.075% solution of hydrogen peroxide. Sections were incubated with peroxidase-conjugated secondary antibodies; rabbit anti-mouse (Dako Denmark A/S, Glostrup, Denmark) for α-SMA and rabbit anti-goat (Dako Denmark A/S) for collagen type I and III. Sections were subsequently incubated with peroxidase-conjugated secondary antibody; goat anti-rabbit (Dako Denmark A/S). Antibody-associated peroxidase activity was visualized with 3-amino-9-ethyl-carbazole (Sigma-Aldrich), and sections were counterstained with Mayer’s hematoxylin. Semiquantitative analysis of liver sections immunostained for either collagen type I and III or α-SMA were performed using the custom Matlab code (www.mathworks.com). For analysis, 10 power fields of a liver section of each rat at 40× magnification were analyzed. The total stained area was set to be equal to the fraction of pixels above a threshold value, typically between 0.950 and 0.965, that left some room for visual matching to the observed staining patterns.

**PAS Staining.** Frozen sections of the livers (4 μm) were fixed in a methanol/formalin (9:1) solution. Subsequently, sections were incubated in 1% periodic acid and after washing in Schiff’s reagent (Merck, Darmstadt, Germany). Nuclei were counterstained with hematoxylin.

PAS staining of liver sections was scored semiquantitatively in a double-blind test, where the following scores were given for the percentage of stained hepatocytes: 1, from 0 to 25% of the liver section was stained for PAS; 2, from 25 to 50% of the liver section was stained for PAS; 3, from 50 to 75% of the liver section was stained for PAS; and 4, from 75 to 100% of the liver section was stained for PAS.

**RNA Isolation and Real-Time RT-PCR Analysis**

Total RNA from cultured HSC was isolated using the Absolutely RNA MicroPrep kit (Stratagene) and from liver tissue with the RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany), both according to the protocol of the manufacturer. The amount of RNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with SuperScript III RNase H-Reverse Transcriptase (Invitrogen, Breda, The Netherlands) and 40 units of RNaseOut inhibitor (Invitrogen) in a volume of 20 μl containing 250 ng of random hexamers (Promega, Madison, WI). Obtained cDNA was diluted with Millipore water (Millipore Corporation, Billerica, MA) to a concentration of 10 ng/μl and 1 μl was applied for each PCR reaction. The following primers purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands) were used for real-time PCR: collagen 1α1 (Rn01463848_m1), TGF-β (Rn00572010_m1), TNF-α (Rn00562055_m1), monocyte chemoattractant protein (MCP)-1 (Rn00580556_m1), and IL-6 (Rn00561420_m1). α-SMA primer was ordered as Assays-by-Design (Applied Biosystems, Foster City, CA) (4331348/assay name ACT-B-ACT2). GAPDH was used as a housekeeping gene (Bodent GAPDH Control Reagent; Applied Biosystems). The PCR reaction was carried out in TaqMan PCR Master Mix (Applied Biosystems) with a final concentration of 200 nM GAPDH primers and 250 nM for primers of the other genes studied. The amplification reaction was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-steps cycles of 15 s at 95°C and 60 s at 60°C. For each sample, the real-time PCR reaction was performed in triplicate, and the averages of the obtained threshold cycle (Ct) values were processed for further calculations according to the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com). In brief, gene expression levels were normalized to the expression of the housekeeping gene GAPDH, giving the ΔCt value. Then, the average value of ΔCt obtained from nontreated HSC or PBS-treated rats was subtracted from the average of the ΔCt value of each sample, yielding the ΔΔCt value. Finally, the gene expression level was calculated as 2−ΔΔCt, giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied genes.

**Statistical Analysis**

Statistical analysis of differences was performed by a two-tailed unpaired Student’s t test. Differences were considered significant when P < 0.05.

**Results**

**Liposomes Characterization.** In M6P-HSA used for the preparation of targeted liposomes, 29 of the 60 ϵ-amino groups of human serum albumin were modified with mannose-6-phosphate moieties.

Liposomes containing POPC were used as control liposomes. Therefore, the lipid composition and lipid molar ratio were the same as in DLPC-liposomes, except that DLPC was
replaced with POPC. Preparations of DLPC and POPC containing liposomes were reproducible, and they yielded liposomes comparable in size as well as in amount of protein coupled (Table 1).

**Effect of DLPC-Containing Liposomes on the Profibrotic Gene Expression Levels in HSC.** The effects of DLPC-containing liposomes on profibrotic gene expression levels were studied in quiescent and activated HSC. During culturing on plastic, HSC spontaneously activate (Friedman et al., 1992). Therefore, we performed experiments with cells cultured for 3 days (HSC d-3) or 10 days (HSC d-10), representing cells with a quiescent or activated phenotype, respectively. The phenotype of the cells was routinely checked to confirm their state of activation. The expression of collagen 1α1 in HSC d-10 increased 4.8 ± 0.94-fold compared with HSC d-3, whereas TGF-β expression was 1.5 ± 0.24-fold higher compared with the expression in HSC d-3. However, mRNA levels of α-SMA were the same in HSC d-10 and HSC d-3.

Both in HSC-3 and HSC-10 substantial inhibition of all three genes that reflect HSC activation was achieved only with the liposomal formulations containing DLPC (Fig. 1). The highest levels of inhibition by DLPC were observed for the M6P-HSA-coupled DLPC-liposomes, especially for the collagen 1α1 and α-SMA genes. Some inhibition of the expression of these genes in the HSC-3 cells was also observed with the M6P-HSA-liposomes without DLPC. In these cells, free M6P-HSA had a small but not significant effect on the expression of collagen 1α1 and α-SMA. Expression of the TGF-β gene was least affected in all cases. Plain liposomes without DLPC had no effect.

To check whether the reduced expression levels of studied genes is not the result of reduced viability of the cells, we studied the influence of DLPC-containing liposomes on the viability of HSC d-3 and HSC d-10. The viability of the cells after 24-h incubation with the various liposomal formulations showed little if any effects by most of liposomal formulations tested (Table 2). A viability decrease was found only for the HSC d-10 cells incubated with untargeted DLPC-liposomes. Incubation with both the M6P-HSA-targeted and the untargeted DLPC-containing formulations caused a small but significant increase in the viability of the HSC d-3. Variation in cell viability can therefore not explain the reduced gene expression levels in these cells.

In a preliminary approach to clarify the mechanism by which the DLPC might exert its down-regulatory effect on profibrotic gene expression, we determined the effect of unesterified linoleic acid on the expression of these genes in the HSC d-3 cells. Incubation with both the M6P-HSA-targeted and the untargeted DLPC-containing formulations caused a small but significant increase in the viability of the HSC d-3. Variation in cell viability can therefore not explain the reduced gene expression levels in these cells.

![Fig. 1. Effects of DLPC-containing liposomes on HSC. Gene expression of TGF-β, collagen 1α1, and α-SMA was determined in HSC d-3 (A) and HSC d-10 (B) using real-time RT-PCR after 24-h incubation with M6P-DLPC-L, DLPC-L, M6P-POPC-L, and POPC-L at a concentration of 320 nmol lipid/ml or 30 μg/ml M6P-HSA as described under Materials and Methods. Expression of the genes by the cells incubated without any addition was set to 1. Data are presented as ± S.E.M. of three to four experiments. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus control.

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<tr>
<th>TABLE 2</th>
<th>Effects of DLPC-containing liposomes on viability of HSC</th>
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<tr>
<td>Viability of Cells</td>
<td>HSC d-3</td>
</tr>
<tr>
<td>Control</td>
<td>100 ± 1</td>
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<tr>
<td>POPC-L</td>
<td>87 ± 1</td>
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<tr>
<td>M6P-HSA-POPCL</td>
<td>101 ± 2</td>
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<tr>
<td>DLPC-L</td>
<td>112 ± 3*</td>
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<tr>
<td>M6P-HSA-DLPC-L</td>
<td>128 ± 4***</td>
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* P < 0.05 versus control. ** P < 0.01 versus control. *** P < 0.001 versus control.

The phenotype of the cells was routinely checked to confirm their state of activation. The expression of collagen 1α1 in HSC d-10 increased 4.8 ± 0.94-fold compared with HSC d-3, whereas TGF-β expression was 1.5 ± 0.24-fold higher compared with the expression in HSC d-3. However, mRNA levels of α-SMA were the same in HSC d-10 and HSC d-3.
Effects of DLPC-Containing Liposomes on Liver Fibrosis in BDL Rats. To examine whether liposomes containing DLPC attenuate the fibrotic processes within the liver, we performed a study using bile duct-ligated rats as a model of liver fibrosis. Measurements of bilirubin, alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transpeptidase serum concentrations confirmed that all rats equally developed an intrahepatic disease process (data not shown). Analysis of profibrotic gene expression levels 24 h after injection of liposomes revealed that in rats treated with untargeted DLPC-containing liposomes, the expression levels of TGF-β and collagen 1α1 were reduced by 70 and 20%, respectively (Fig. 2). In contrast, M6P-HSA DLPC-liposomes slightly up-regulated the expression of collagen 1α1 and α-SMA, whereas TGF-β expression was not affected.

Expression of collagen types I and III as well as of α-SMA in BDL rats treated with liposomes was also investigated at the protein level. Immunohistochemical analysis of liver sections showed a reduction of collagen type I and III and α-SMA by 40 and 30%, respectively, in rats treated with untargeted DLPC-liposomes compared with PBS-treated animals. None of the other liposomal formulations significantly affected the expression level of either collagen or α-SMA (Fig. 3).

Because prolonged damage to hepatocytes initiates the fibrotic process in the liver, we investigated the effect of different liposomes on these cells in the fibrotic livers of BDL rats. Detection of glycogen stored in hepatocytes showed that the number of hepatocytes containing glycogen in the livers of rats treated with DLPC liposomes and M6P-HSA-DLPC-liposomes was higher than in control rats injected with PBS (Fig. 4). Control liposomes did not induce any effect on glycogen storage in hepatocytes.

Effects of DLPC-Containing Liposomes on Proinflammatory Gene Expression in Livers of BDL Rats and Primary Cultured Kupffer Cells and Liver Endothelial Cells. Our previous studies on the uptake of M6P-HSA-targeted liposomes by hepatic cells in fibrotic livers showed that they not only accumulate in HSC but also in KC and LEC. We hypothesized that the accumulation of M6P-HSA-targeted DLPC-liposomes in KC and LEC may activate these cells and stimulate the fibrotic process. To investigate this, we analyzed the expression of proinflammatory cytokines such as TNF-α, MCP-1, and IL-6 in BDL rats treated with liposomes. Expression levels of MCP-1 and TNF-α were up-regulated 145 and 25%, respectively, by M6P-HSA-DLPC-liposomes (Fig. 5A). Interestingly, in rats injected with DLPC-liposomes, mRNA level of TNF-α was down-regulated by 30% compared with untreated rats.

We also tested the influence of DLPC-containing liposomes...
on TNF-α, MCP-1, and IL-6 expression by primary cultured rat KC and LEC. After 24-h incubation of these cells with liposomes, we observed that M6P-HSA-DLPC-liposomes had significantly decreased the expression of MCP-1 and TNF-α in KC, whereas a 4-fold up-regulation of IL-6 was observed in these cells (Fig. 5B). Likewise, M6P-HSA-DLPC-liposomes caused up-regulation of IL-6 in LEC (Fig. 5C). Expression levels of TNF-α, MCP-1, and IL-6 in KC and LEC were down-regulated by DLPC-liposomes. M6P-HSA-POPC-liposomes stimulated IL-6 and MCP-1 expression in both KC and LEC, whereas POPC-liposomes inhibited expression of the genes studied. So, whereas DLPC and POPC liposomes reduced expression of TNF-α, MCP-1, and IL-6 in KC and LEC, coupling of M6P-HSA to these liposomes increased the expression levels most of these proinflammatory genes.

**Discussion**

DLPC is the major compound of polyenylphosphatidylcholines extracted from soybean (*Glycine max*) and has been shown to be biologically active (Lieber et al., 1994; Ma et al., 1996). Studies in animal models of liver fibrosis showed that supplementation of the food with polyenylphosphatidylcholines has beneficial effects on the fibrotic liver. In addition, it was demonstrated on cultured HSC that DLPC reduces the activation of HSC by down-regulating α-SMA and α1(I) pro-collagen gene expression (Poniachik et al., 1999; Cao et al., 2002c) and that it inhibits HSC proliferation (Brady et al., 1998). Recently, it was reported that DLPC prevents production of tissue inhibitor of matrix metalloproteinase 1 in a human hepatic stellate cell line (Cao et al., 2006). Moreover, DLPC decreases TNF-α production in KC isolated from ethanol-fed rats (Cao et al., 2002a,b). In these studies, DLPC was shown to act as an antioxidant and to interfere at the molecular level with mitogen-activated protein kinases signaling pathways in HSC and KC.

By incorporating the bioactive phospholipid DLPC in the bilayer of liposomes, we made an attempt to design a drug carrier system that, when properly targeted, could simultaneously deliver an encapsulated antifibrotic drug as well as the bioactive lipid to the HSC population in fibrotic livers. Because liver fibrosis is a complex disease involving activation of HSC via different pathways, most probably more than one therapeutic compound needs to be delivered to the liver.

Bioactive drug carriers may provide such opportunity. To target our carrier to HSC in the fibrotic liver, we coupled M6P-HSA to the surface of liposomes (Adrian et al., 2006).

In our experiments, M6P-HSA-DLPC- and DLPC-liposomes, but not POPC-containing liposomes, induced a strong down-regulation of profibrotic genes such as TGF-β, collagen 1α1, and α-SMA in quiescent and activated HSC. This confirmed the reported antifibrotic properties of DLPC and demonstrated that the incorporation into liposomes did not affect DLPC activity. Since free linoleic acid (LA) attenuated the expression of TGF-β, collagen 1α1, and α-SMA in HSC, it is plausible that the observed effects of the liposomes are due to the presence of DLPC, with LA as the main active group.

In HSC day 3, free M6P-HSA- and M6P-HSA-POPC-liposomes also slightly reduced expression of collagen 1α1 and α-SMA but not of TGF-β. Since the expression level of M6P/ IGF II receptor is very low in HSC day 3 (Adrian et al., 2006), this may be caused by competition between M6P-HSA and inactive TGF-β for the receptor. As a result the activation of TGF-β and TGF-β effects on α-SMA and collagen 1α1 may be inhibited. In HSC day 10, the M6P/IGF II receptor is overexpressed to an extent that M6P-HSA may not affect the activation of TGF-β.

The mechanism by which DLPC-containing liposomes affect the fibrotic process in vivo is less straightforward. Surprisingly, at the mRNA level, M6P-HSA-DLPC-liposomes did not affect (TGF-β) or even slightly enhanced the expression (collagen 1α1 and α-SMA) of profibrotic genes. In contrast, DLPC-liposomes significantly reduced gene expression of two important fibrosis markers, TGF-β and collagen 1α1. Immunohistochemical staining of collagen I and III as well as α-SMA in rats treated with DLPC-liposomes showed a reduction in protein expression as well. These findings demonstrated that DLPC incorporated into liposomes can modulate the fibrotic process in the liver, whereas M6P-HSA might influence this effect in vivo.

In addition to HSC, also KC and LEC take up M6P-HSA-liposomes via a scavenger receptor-mediated pathway. Both KC and LEC are known to play a major role in inflammatory processes in the liver. Injury to hepatocytes causes activation of KC that respond by producing, among others, proinflammatory cytokines such as TNF-α, IL-1, IL-6, and TGF-β. This latter cytokine is a key stimulus for HSC to produce extracellular matrix components (Ramadori and Armbrust, 2001).
TNF-α induces expression of cell adhesion molecules in LEC, which triggers the recruitment of inflammatory cells. In addition, TNF-α has a direct effect on HSC and induces secretion of leukocyte chemoattractants such as MCP-1, macrophage-inhibitory protein 2 (Sprenger et al., 1999), and cytokine-induced neutrophil chemoattractant (rat analog of IL-8) (Maher et al., 1998) as well as expression of leukocyte adhesion markers such as intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Kniittel et al., 1999). Therefore, we hypothesized that, due to the uptake of M6P-HSA-DLPC-liposomes by KC and LEC in fibrotic livers, the activation status of these cells may be modulated and even may promote progression of fibrotic process, which would counteract the beneficial effects of DLPC. Comparison of the effects of DLPC-containing liposomes on MCP-1, IL-6, and TNF-α gene expression in fibrotic livers and primary cultures of KC and LEC did not produce a clear picture. Although in fibrotic livers these genes were up-regulated by M6P-HSA-DLPC-liposomes, only the levels of IL-6 were elevated in cultured KC and LEC, whereas the expression of MCP-1 and TNF-α was suppressed. The response of cultured KC and LEC is likely to be different from that of activated KC and LEC in fibrotic livers. Discrepancies between the effects of DLPC-containing liposomes in vitro and in vivo may therefore be readily explained. To achieve a more selective liposome delivery to HSC, a different homing ligand to these cells could be coupled to the surface of DLPC-containing liposomes. For example, human serum albumin modified with a peptide that is recognized by platelet-derived growth factor receptor on HSC (Beljaars et al., 2003) is an alternative option here, because uptake of this protein construct in other nonparenchymal cells is minimal.

The loss of hepatocytes in fibrotic livers and the impaired functionality of those that are still present are well known characteristics of liver fibrosis. The ability of these cells to store glycogen reflects their overall condition. Therefore, estimation of the glycogen content of hepatocytes may serve as a measure of damage within the liver. Reduced glycogen storage has been reported in patients with alcohol-induced and biliary liver cirrhosis (Krahenbuhl et al., 2003) as well as in rat CCl₄ (Krahenbuhl et al., 1991) and BDL (Krahenbuhl et al., 1996) models of the disease. The mechanism leading to the reduced hepatic glycogen storage is not fully understood, but reduced volume of hepatocytes and the altered hepatocellular metabolism of glycogen probably play a role here. Interestingly, in our experiments we observed a restoration of the hepatocyte glycogen content in fibrotic rats treated with DLPC-containing liposomes. Part of the injected dose of DLPC-liposomes may be taken up by hepatocytes, because these are neutral liposomes, smaller than 100 nm, and they are therefore able to pass the fenestration in endothelial lining of the sinusoids (Scherphof and Kamps, 2001). However, the effect of M6P-HSA-DLPC-liposomes on hepatocytes is likely to be a secondary response to lipids derived from liposomes taken up by target cells. Liposomes containing phosphatidylcholine enter the cells via endocytosis (Adrian et al., 2006). For example, human serum albumin modified with a peptide that is recognized by platelet-derived growth factor receptor on HSC (Beljaars et al., 2003) is an alternative option here, because uptake of this protein construct in other nonparenchymal cells is minimal.

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Fig. 5. Comparison of the effects of DLPC-containing liposomes on proinflammatory gene expression in fibrotic livers and primary cultures of KC and LEC. A, gene expression of MCP-1, IL-6, and TNF-α in livers of BDL rats injected with different liposomal formulations: M6P-HSA-DLPC-L, DLPC-L, M6P-HSA-POPC-L, and POPC-L at a dose of 3 μmol of lipid per kilogram of body weight. Gene expression levels in rats treated with PBS were set at 1. Data are presented as ± S.E.M. of four to five rats. *, P < 0.05; **, P < 0.001 versus control. Gene expression of MCP-1, IL-6, and TNF-α was measured in KC (B) and LEC (C) using real-time RT-PCR after 24-h incubation with M6P-HSA-DLPC-L, DLPC-L, M6P-HSA-POPC-L, and POPC-L at the concentration of 320 nmol lipid/ml as described under Materials and Methods. Expression of the genes by KC or LEC incubated without liposomes was set at 1. Data are presented as ± S.E.M. of three experiments. *, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus control.
patients with liver cirrhosis, PUFA deficiency is a well-described phenomenon, but so far the cellular and clinical sequence of events has been not fully elucidated (Johnson et al., 1985; Clemmensen et al., 2000). PUFA are also responsible for the proper functions of the cell membrane by maintaining its fluidity and the proper activity of the membrane-bound proteins. LA-derived PUFA such as dihomo-gamma-linolenic acid and arachidonic acid are precursors of eicosanoids. Finally, LA and LA-derived molecules are natural activators of peroxisome proliferator-activated receptors (Clement et al., 2002), transcription factors that influence intracellular lipids and carbohydrates metabolism through direct transcriptional control of genes involved in peroxisomal and mitochondrial β-oxidation pathways, fatty acids uptake, and triglyceride metabolism. In addition, activation of peroxisome proliferator-activated receptors has been described to regulate anti-inflammatory responses (Chinetti et al., 2000), and LA was reported to be protective against hepatocytes injury (Li and Akaike, 1998). Overall, these observations suggest that DLPC-liposomes, due to their high LA content, display profound effects in hepatocytes leading to hepatoprotective effects in livers of BDL rats. However, the precise mechanisms via which LA influence cells in the fibrotic liver need to be elucidated.

In conclusion, we successfully incorporated DLPC into the membranes of liposomes targeted to HSC. In cultured HSC, these bioactive drug carriers strongly reduced the expression of the genes for collagen Iα and α-SMA, important markers of fibrosis. In the fibrotic livers of BDL rats, the uptake of M6P-HSA-DLPC-liposomes in KC and LEC might activate these cells and counteract the positive effects of DLPC in HSC, whereas uptake of DLPC in hepatocytes seems to be beneficial. The use of bioactive drug carrier in fibrotic liver is feasible and promising for a complex disease such as fibrosis. However, cell selectivity of the carrier is crucial for this approach.

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