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Research Paper

Targeting 15d-Prostaglandin J_2 to Hepatic Stellate Cells: Two Options Evaluated

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Purpose. Delivery of apoptosis-inducing compounds to hepatic stellate cells (HSC) may be an effective strategy to reverse liver fibrosis. The aim of this study was therefore to examine the selective targeting of the apoptosis-inducing drug 15-deoxy-Δ12,14-prostaglandin J_2 (15dPGJ$_2$) with two different HSC-carriers: human serum albumin modified with the sugar mannose-6-phosphate (M6PHSA) or albumin modified with PDGF-receptor recognizing peptides (pPBHSA).
Methods and Results. After chemical conjugation of 15dPGJ$_2$ to the carriers, the constructs displayed pharmacological activity and specific receptor-mediated binding to HSC *in vitro*. Unlike 15dPGJ$_2$-pPBHSA, the cellular binding of 15dPGJ$_2$-M6PHSA was reduced by a scavenger receptor antagonist. *In vivo*, both conjugates rapidly accumulated in fibrotic livers. Intrahepatic analysis revealed that 15dPGJ$_2$-M6PHSA mainly accumulated in HSC, and to a lesser extent in Kupffer cells. 15dPGJ$_2$-pPBHSA also predominantly accumulated in HSC with additional uptake in hepatocytes. Assessment of target receptors in human cirrhotic livers revealed that M6P/IGFII-receptor expression was present in fibrotic areas. PDGF-β receptor expression was abundantly expressed on human fibroblasts.
Conclusions. These studies show that 15dPGJ$_2$ coupled to either M6PHSA or pPBHSA is specifically taken up by HSC and is highly effective within these cells. Both carriers differ with respect to receptor specificity, leading to differences in intrahepatic distribution. Nevertheless, both carriers can be used to deliver the apoptosis-inducing drug 15dPGJ$_2$ to HSC *in vivo*.

KEY WORDS: antifibrotic drugs; drug targeting; hepatic stellate cells; liver fibrosis; 15dPGJ$_2$.

INTRODUCTION

Liver fibrosis may occur as a response to chronic liver injury and may lead to cirrhosis. HSC play a pivotal role in this fibrogenic process, because this cell starts to proliferate and accumulate within the injured liver. During this activation process, increased amounts of collagen and other extracellular matrix compounds and inhibitors with matrix degrading activity are produced by these HSC, thereby strongly affecting the liver architecture and eventually liver function (1,2). Up till now, no pharmaceutical intervention is available to treat this fibrotic disease (3). The application of antifibrotic drugs has not been successful, partly because these drugs do not accumulate in the target cells in the diseased liver or cause side effects elsewhere in the body. Alteration of the pharmacokinetic characteristics of the drug by means of drug targeting may represent a promising approach in the development of an effective antifibrotic drug.

In recent years, different HSC-selective carrier systems have become available (4–6). One system uses the sugar mannose-6-phosphate which is coupled to the protein human serum albumin to form M6P$_{28}$HSA. This carrier binds to mannose-6-phosphate/insulin-like growth factor-II (M6P/IGFII) receptors (4,7–10). The expression of these M6P/IGFII receptors increases as a result of HSC activation during liver fibrosis (11) enabling selective accumulation of M6PHSA in HSC of fibrotic rats (4).

In other delivery systems for HSC, the homing devices are cyclic receptor-recognizing peptides (5,6). The carrier homing to the PDGF-β receptor (pPBHSA) may be quite relevant, since PDGF-BB is the major cytokine involved in the proliferation of HSC during the fibrotic process, and the PDGF-β receptor is highly upregulated on activated HSC

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ABBREVIATIONS: BDL, bile duct ligation; FCS, fetal calf serum; HSA, Human Serum Albumin; HSC, hepatic stellate cells; M6P/IGFII receptor, Mannose-6-Phosphate/Insulin-like Growth Factor-II receptor; M6PHSA, human serum albumin modified with the sugar mannose-6-phosphate; PDGF-β receptor, platelet derived growth factor beta receptor; pPBHSA, human serum albumin modified with PDGF-receptor recognizing peptides; 15dPGJ$_2$, 15d-prostaglandin J_2.

(12,13). The carrier pPBHSA competitively inhibited the binding of PDGF-BB to its receptor, and *in vivo* preferentially homed to the HSC in fibrotic rat livers with minor uptake in hepatocytes (5). *In vivo*, uptake was associated with disease-induced expression of the PDGF receptor in liver and kidney.

In experimental liver fibrosis, resolution of the disease was found to be accompanied by loss of HSC via spontaneous induction of apoptosis in these cells (14,15). Therefore, induction of apoptosis in HSC might represent an attractive approach to reverse this liver disease. In this respect, 15dPGJ$_2$ is interesting, because it was shown that this prostaglandin induced apoptosis of human hepatic myofibroblasts (16). In addition, sub-apoptotic concentrations of 15dPGJ$_2$ significantly inhibited the expression of interstitial collagens as well as its proliferation in human hepatic myofibroblasts *in vitro* (17,18). 15dPGJ$_2$ was also found in vivo at sites of inflammation during the resolution phase of the disease, suggesting that it might function as a negative feedback regulator of the inflammatory process (19).

However, generally the pharmacokinetic properties of prostaglandins limit their therapeutic use. Prostaglandins are locally acting mediators that are rapidly metabolized by the lung (20) or in plasma (21) and excreted from the body via the kidneys resulting in a very short half life (22). For example, prostaglandin E$_2$ (dinoprostone) has a plasma half-life ($t_{1/2}$) of less than 1 min and prostaglandin I$_2$ (epoprostenol) has a $t_{1/2}$ of 2–3 min (23). The $t_{1/2}$ of 15dPGJ2 is not known. In addition, the high protein binding of prostaglandins in serum (24) might prevent 15dPGJ$_2$ to effectively reach the essential cells within the liver after systemic administration. In line with this, *in vitro* studies demonstrated that pharmacological effects of 15dPGJ$_2$ were abolished in the presence of serum (16). Specific delivery of prostaglandins to HSC might overcome these problems.

The aim of this study was therefore to chemically couple 15dPGJ$_2$ to two different HSC-selective drug carriers, i.e., M6PHSA and pPBHSA, and to compare their effectiveness by studying the uptake and pharmacological effects within the target cells.

MATERIAL AND METHODS

Materials

HSA was purchased from the Central Laboratory of Blood Transfusion Services (Sanquin Blood Supplies, Amsterdam, The Netherlands). 15dPGJ$_2$ was purchased from ITK Diagnostics (Uithoorn, The Netherlands). All other chemicals used were of analytical grade.

Animals

Wistar rats (male, outbred strain, obtained from Harlan, Horst, The Netherlands) were housed under standard laboratory conditions and had free access to food and water. This study was performed in accordance with ethical regulations imposed by Dutch legislation.

Synthesis

M6PHSA. HSA was modified with mannose-6-phosphate groups as described (4). The monomeric fraction was separated from the dimeric and polymeric fraction by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Uppsala, Sweden) in an FPLC system. The monomeric protein was characterized for protein, sugar, and phosphate content.

pPBHSA. The cyclic peptide C*SRNLIDC* was prepared by Ansynth Service BV (Roosendaal, The Netherlands) and covalently coupled to HSA as described (5).

Conjugation of 15dPGJ2 to the Albumin Carriers. The carboxylic acid group of 15dPGJ$_2$ (10 mg; 32 μmol) dissolved in 150 μl dimethylformamid (DMF)) was activated with 12.5 mg (61 μmol) N,N-dicyclohexylcarbodiimide (DCC, Sigma, St. Louis, MO, USA), in a ratio 15dPGJ2:DCC = 1:2, dissolved in 80 μl DMF and stirred for 1 h (RT). This 15dPGJ$_2$/DCC solution was subsequently added to 20 mg (0.25 μmol) M6PHSA or pPBHSA (dissolved in PBS, 4 mg/ml) in ratio 15dPGJ2:carrier = 130:1, and the mixture was stirred at RT during 16 h. The obtained solutions were extensively dialyzed (Slide-A-Lyzer 10K, Pierce Biotechnology, Rockford, IL, USA) against milliQ water at 4°C for 2 days followed by overnight dialysis against 5 mg/ml HSA solution to remove all low molecular weight compounds and unbound 15dPGJ$_2$. The conjugates were lyophilized and stored at −20°C. The amount of 15dPGJ$_2$ coupled to the carrier was assessed using the 15dPGJ$_2$ correlate-EIA kit (Assay Designs, Ann Arbor, Michigan, USA).

Rat Hepatic Stellate Cell Isolation

We isolated primary HSC from livers of rats (±500 g body weight) using *in situ* perfusion and subsequent digestion of the livers with collagenase P, DNAse (both Roche Diagnostics, Indianapolis, IN, USA) and pronase E (Merck, Darmstadt, Germany). After separation of the HSC from other hepatic cells by density-gradient centrifugation, the HSC were collected on top of a 12% Nycodenz-solution (25). Subsequently, the cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), and incubated in a 5% CO_2 humidified atmosphere at 37°C. Cells cultured for 10 days were used for further experiments. At this time point, all cells have an activated phenotype. This was routinely microscopically evaluated and regularly checked by staining for α-smooth muscle actin using a monoclonal antibody (clone 1A4, Sigma).

Binding to HSC

To assess the cellular binding of the conjugates as described (7), 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA were radioactively labelled with ^{125}I (26).

One hundred thousand cells (10 days after isolation) were grown in a six well-plate and pre-incubated with 1% bovine serum albumin in DMEM to block non-specific binding. HSC were then incubated at 37°C with 100,000 cpm of ^{125}I-labelled conjugate in DMEM with 0.2% bovine serum albumin. In addition, wells were co-incubated with

HSA, M6PHSA, pPBHSA (all 1 mg/ml) or polyinosinic acid (PIA, 10 μg/ml). After 2 h, cells were washed with PBS and the cell-associated radioactivity was measured on a γ-counter (Riastar, Packard instruments, Palo Alto, USA). These experiments were performed with cells of three separate HSC isolations.

Biological Effects on HSC

HSC (day 7 after isolation; 5,000/well) were incubated in a 96-well plate for 24 h in 200 μl medium with serum, then washed with serum-free medium and incubated for an additional 24 h in 200 μl medium with or without 10% FCS. Subsequently, $15dPGJ_2$, $15dPGJ_2$-M6PHSA, M6PHSA, $15dPGJ_2$-pPBHSA or pPBHSA were added in increasing concentrations and the cells were incubated with or without 10% FCS for another 18 h. Alamar blue (20 μl, Serotec, Oxford, UK) was added and the cells were incubated for an additional 24 h (i.e., final analysis of HSC at day 10 after isolation). The conversion of Alamar blue by the metabolic activity of the cells reflects the number of cells (i.e., net result of proliferation and apoptosis) present in each well. The Alamar blue conversion was measured with a fluorimeter (27). These experiments were performed with cells of three separate HSC isolations.

Animal Model of Liver Fibrosis

To induce liver fibrosis, rats (220–240 g) were subjected to bile duct ligation (BDL) under isoflurane/O_2/N_2O anesthesia. The rats were used for further experiments at 10 days after ligation, when HSC are activated and excess of extracellular matrix deposits are clearly present (portal expansion with occasional portal-to-portal bridging).

Organ Distribution

BDL rats were injected intravenously, in the penile vein, with a tracer dose of ^{125}I-HSA, ^{125}I–$15dPGJ_2$-M6PHSA or ^{125}I–$15dPGJ_2$-pPBHSA (1.10^6 cpm per rat, $n = 3$/group) under anaesthesia. Fifteen minutes after injection, the animals were sacrificed, blood was obtained by heart puncture and organs were removed. The radioactivity in each organ was measured with a γ-counter (Packard instruments). The total radioactivity measured in the total organ was corrected for the blood-derived radioactivity according to standard procedures (6).

Hepatic Localization

Anaesthetized BDL rats were injected intravenously with 2 mg/kg $15dPGJ_2$-M6PHSA or $15dPGJ_2$-pPBHSA ($n = 3$/group). Fifteen minutes after administration, the rats were sacrificed and sections of the liver were frozen in isopentane (−80°C).

Immunohistochemical analysis of acetone-fixed cryostat sections (4 μm) of the livers was used to localize the carrier. Therefore, livers were double-stained with a polyclonal antibody against HSA (MP Biomedicals, Zoetermeer, The Netherlands) combined with an antibody specific for either the Kupffer cells (ED2, Serotec), sinusoidal endothelial cells (RECA1, Serotec) or HSC (Desmin and Glial Fibrillary Acid Protein (GFAP) simultaneously, both Sigma) (4). The double-stained sections were quantified by two independent observers (at least ten microscopical fields at 20×10 magnification). The total number of double positive cells (red staining reflects HSA-positive cells and blue staining reflects the cell marker) was counted and divided by the total number of HSA-positive cells (all red staining) in the same microscopical area. This yielded the relative uptake of the conjugate by each cell-type.

Localisation of Target Receptors in Human Tissue

Acetone-fixed cryostat sections (4 μm) of human livers from cirrhotic patients ($n = 3$) and healthy human livers, obtained from donor livers ($n = 3$), were stained according to standard immunohistochemical techniques (28). The primary antibodies used in this study were polyclonal antibodies directed against the M6P/IGFII-receptor (K-21) and PDGF-β receptor (958) (both Santa Cruz Biotechnology, CA, USA). After incubation with two secondary antibodies, amino-ethyl carbazole (AEC) was used to visualize the staining.

The scientific use of human liver tissue was approved by the Medical Ethical Committee of the University Medical Center Groningen (The Netherlands).

Statistics

Results are expressed as ±SEM. Statistical analysis was performed by two tailed Student's *t*-test. *P* values lower than 0.05 were considered statistically significant.

RESULTS

$15dPGJ_2$ was chemically linked via its COOH-group to the free NH2-group of the lysines as present in M6PHSA and pPBHSA. Using ELISA techniques to detect $15dPGJ_2$, we assessed the presence of $15dPGJ_2$ in the lyophilized and

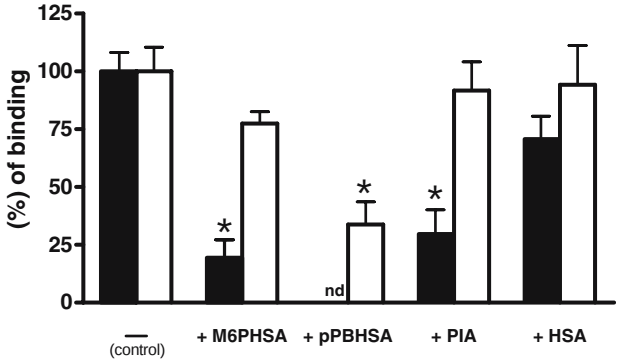

Fig. 1. Binding of the conjugates ^{125}I–$15dPGJ_2$-M6PHSA (*black bars*) and ^{125}I–$15dPGJ_2$-pPBHSA (*white bars*) to cultures of activated rat HSC. Incubation of ^{125}I-labelled conjugate and excess amounts of unlabeled M6PHSA, pPBHSA, the scavenger receptor antagonist polyinosinic acid (PIA), and HSA was performed to assess receptor-specific uptake of the conjugates by the HSC. The results of separate experiments with three different HSC isolations are expressed as the mean ± SEM (* = $P < 0.05$ as compared to control bar). *nd* = not determined.

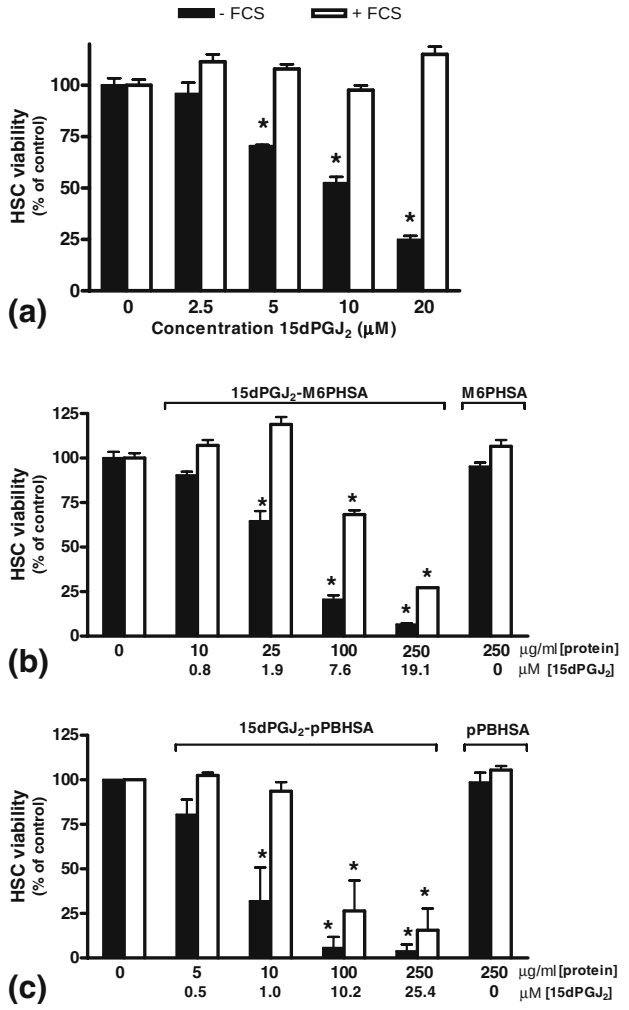

Fig. 2. Effect of different 15dPGJ$_2$ forms on the viability of isolated activated HSC. (a) 15dPGJ$_2$, (b) 15dPGJ$_2$-M6PHSA and the carrier M6PHSA control, (c) 15dPGJ$_2$-pPBHSA and the carrier pPBHSA control. The experiments were performed under serum starvation (*black bars*) or in the presence of 10% FCS (*white bars*). Of both conjugates, the estimated concentration of 15dPGJ$_2$ present in 15dPGJ$_2$-M6PHSA (6 molecules drug/protein) or 15dPGJ$_2$-pPBHSA (8 molecules drug/protein) is depicted as well as their protein concentrations. The control cells (incubated with vehicle) are set at 100% viability and control wells without cells are set as 0% viability. The results are expressed as the mean ± SEM ($n = 3$, * = $P < 0.05$).

purified conjugates, while the carriers alone did not give an ELISA-signal. We calculated that, respectively, six and eight molecules 15dPGJ$_2$ were coupled to M6PHSA and pPBHSA. Subsequently, we confirmed that after conjugation of the carriers with the lipophilic drug 15dPGJ$_2$, the ability of both conjugates to bind rat HSC was preserved. Radioactive studies showed significant binding of both constructs to HSC *in vitro* (Fig. 1). To confirm that the cellular internalization of 15dPGJ$_2$ conjugated with M6PHSA or pPBHSA occurred via two different receptors, i.e., respectively, M6P/IGFII receptor and PDGFβ-receptor, we co-incubated the radioactive labeled conjugates with excess amounts of unlabeled M6PHSA or pPBHSA. For these studies, we used tracer amounts of the radioactive compounds, because tracer doses are representative for all non-saturated dosages. The negatively charged molecule polyinosinic acid, a well-known antagonist of scavenger receptors (29), was included in these studies because coupling of approximately 33 M6P groups to albumin significantly increases the net negative charge of the protein, which implicates that the scavenger receptor might be involved in the uptake of M6PHSA by HSC.

In Fig. 1 it can be seen that binding of ^{125}I–15dPGJ$_2$-M6PHSA to HSC was significantly reduced by 81 ± 5% ($P < 0.05$) after co-incubation with an excess of M6PHSA, a ligand for the M6P/IGFII receptor. Excess of HSA alone did not significantly reduce the binding of ^{125}I–15dPGJ$_2$-M6PHSA to the HSC. Co-incubation with polyinosinic acid resulted in a reduction of cellular binding by 70 ± 10% ($P < 0.05$). This indicates that the binding of 15dPGJ$_2$-M6PHSA to target cells is mediated by M6P/IGFII receptor and scavenger receptors present on the cell membrane.

The receptor-mediated binding of the other carrier, ^{125}I–15dPGJ$_2$-pPBHSA, was significantly reduced with an excess of pPBHSA. This protein reduced the binding of 15dPGJ$_2$-pPBHSA to HSC by 66 ± 10% ($P < 0.05$). Neither co-incubation with HSA nor M6PHSA or polyinosinic acid reduced the binding of ^{125}I–15dPGJ$_2$-pPBHSA to the HSC, indicating the specificity of its binding to the cell membrane. These studies show that the binding and uptake of 15dPGJ$_2$ coupled to the carrier M6PHSA occurs via different cell membrane receptors than 15dPGJ$_2$ coupled to pPBHSA.

Induction of Apoptosis

We subsequently examined the apoptosis-inducing effect of 15dPGJ$_2$ in its free form and conjugated to M6PHSA or pPBHSA by measuring the viability of primary isolated rat HSC. We also incorporated the influence of serum, since serum components such as albumin are known factors that destroy the bioactivity of prostaglandins (24). This aspect is of course quite relevant *in vivo*. Addition of native 15dPGJ$_2$ to cells grown without FCS caused a significant and dose-dependent decline in viability (80% reduction at 20 μM 15dPGJ$_2$). Co-incubation of 15dPGJ$_2$ with 10% FCS completely abolished the activity of 15dPGJ$_2$ in cell cultures (Fig. 2a). Subsequently, we examined the effects of both conjugates. Without FSC, both conjugates induced a dose-dependent reduction in the viability of HSC starting at a concentration

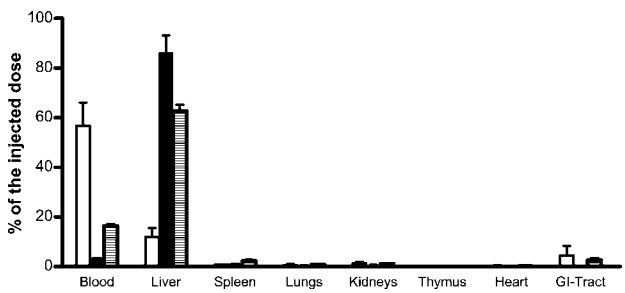

Fig. 3. *In vivo* organ distribution of ^{125}I-labeled 15dPGJ$_2$-M6PHSA (*black bars*), 15dPGJ$_2$-pPBHSA (*striped bars*), and HSA (*white bars*) at 15 min after intravenous injection of the compounds in rats with BDL-induced liver fibrosis.

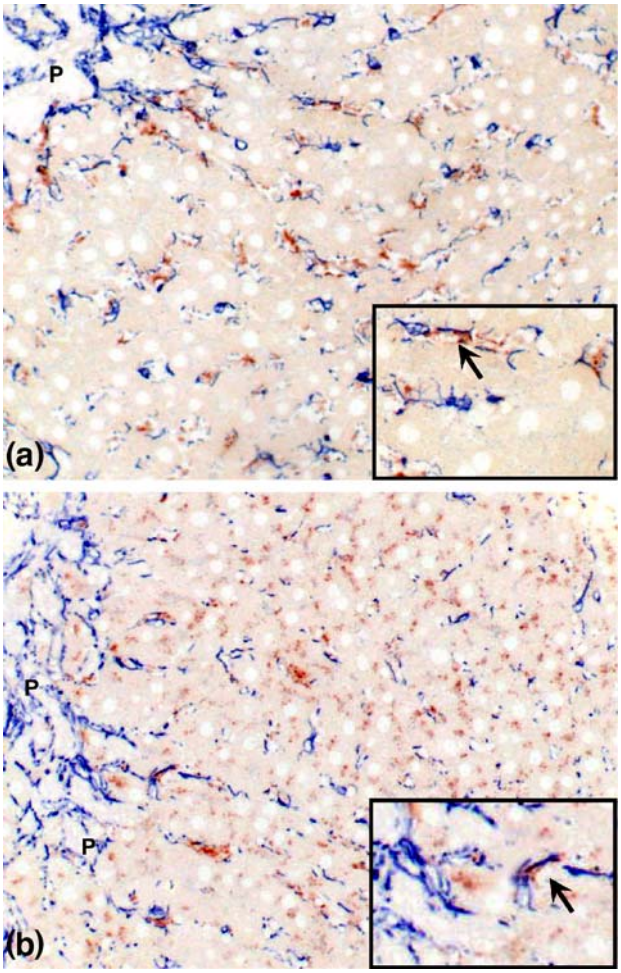

Fig. 4. Immunohistochemical localization of 15dPGJ$_2$-M6PHSA (a) and 15dPGJ$_2$-pPBHSA (b) in liver of BDL-10d rats, 15 min after intravenous injection of the constructs. Cell-specific localization was determined by double staining of the livers with an antibody directed against HSA (*red* staining) together with cellular markers to detect HSC (desmin and GFAP; *blue* staining). Magnification 200×. The inserts shows co-localization of the modified HSA together with HSC (*arrow*; magnification 400×). *P* = portal area.

of 25 μg/ml 15dPGJ$_2$-M6PHSA (corresponding with 1.9 μM 15dPGJ$_2$) and 10 μg/ml 15dPGJ$_2$-pPBHSA (corresponding with 1.0 μM 15dPGJ$_2$). It may be derived from Fig. 2a and b that 15dPGJ$_2$ targeted with pPBHSA is somewhat more potent than 15dPGJ$_2$ targeted with M6PHSA since lower concentrations of conjugated drug result in higher efficacy. However, additional studies are needed to prove this. In the presence of 10% FCS, both conjugates were still able to reduce the HSC viability, although the dose–response curve was somewhat shifted to the right (Fig. 2b, 69% reduction at 250 μg/ml 15dPGJ$_2$-M6PHSA; Fig. 2c, 81% reduction at 250 μg/ml 15dPGJ$_2$-pPBHSA). This small shift is most likely due to a serum effect on cells, i.e., in the presence of serum these cells are more resistant to apoptotic stimuli. When the carriers alone (250 μg/ml of M6PHSA or pPBHSA) were incubated with the HSC cultures no influence on cell viability in both serum and serum-free conditions were measurable (Fig. 2b and c).

Body Distribution in Rats with Liver Fibrosis

The organ distribution of HSA, 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA was studied in BDL rats, 10 days after induction of the disease (Fig. 3). Fifteen minutes after intravenous administration, both ^{125}I–15dPGJ$_2$-M6PHSA and ^{125}I–15dPGJ$_2$-pPBHSA accumulated significantly in the livers (86 and 63% of the dose, respectively), leaving little conjugate in the blood (3 and 16%, respectively). Less than 3% of the administered ^{125}I–15dPGJ$_2$-M6PHSA and only 7% of the ^{125}I–15dPGJ$_2$-pPBHSA dose was taken up in other organs and tissues. In contrast, organ distribution studies of ^{125}I–HSA in BDL-10d rats revealed that the majority of the injected dose was present in the blood at 15 min after injection (Fig. 3). In conclusion, these data indicate a rapid and almost complete distribution of both conjugates to the fibrotic liver.

To determine the cellular specificity of 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA within the diseased livers, we examined the intrahepatic distribution of the conjugates in BDL-10d rats with immunohistochemical techniques (Fig. 4). 15dPGJ$_2$-M6PHSA was clearly taken up by non-parenchymal cells and co-localization with HSC markers was found (Fig. 4a). Quantitative evaluation by counting double-positive cells showed that 64% of the double-positive cells could be identified as HSC (Table I). The second cell type that displayed substantial uptake of the M6P-conjugate was the Kupffer cell (39%). Quantitative evaluation of the cellular distribution of 15dPGJ$_2$ targeted with the other carrier pPBHSA revealed that 57% of the double-positive non-parenchymal cells were identified as HSC (Table I). For this carrier, the hepatocytes also contributed to the uptake of 15dPGJ$_2$-pPBHSA in the fibrotic liver, but our scoring procedure did not allow good quantification of this diffuse staining.

M6P/IGFII and PDGF-β Receptor Staining in Normal and Cirrhotic Human Livers

Finally, we examined the expression of the target receptors for our HSC-specific carriers in histologically normal and cirrhotic human livers (Fig. 5). Normal liver sections displayed a parenchymal staining for the M6P/IGFII receptor. In cirrhotic human livers, M6P/IGFII-receptor

Table I. Estimation of the Intrahepatic Distribution of 15dPGJ$_2$-M6PHSA and 15dPGJ$_2$-pPBHSA in BDL-10d Fibrotic Rat Livers 15 min after Intravenous Injection

	15dPGJ2-M6PHSA	15dPGJ2-pPBHSA
Hepatic Stellate Cells (%)	64 ± 5	57 ± 7
Kupffer Cells (%)	39 ± 3	24 ± 8
Sinusoidal Endothelial Cells (%)	7 ± 5	32 ± 7
Hepatocytes	−	+

The contribution of each hepatic cell type in the total liver uptake of the conjugate was determined by counting the number of double-positive cells (HSA+ and cell marker+) relative to the total number of conjugate-positive cells in the same microscopical field. Values are expressed as the percentage double-positive cells ± SD.

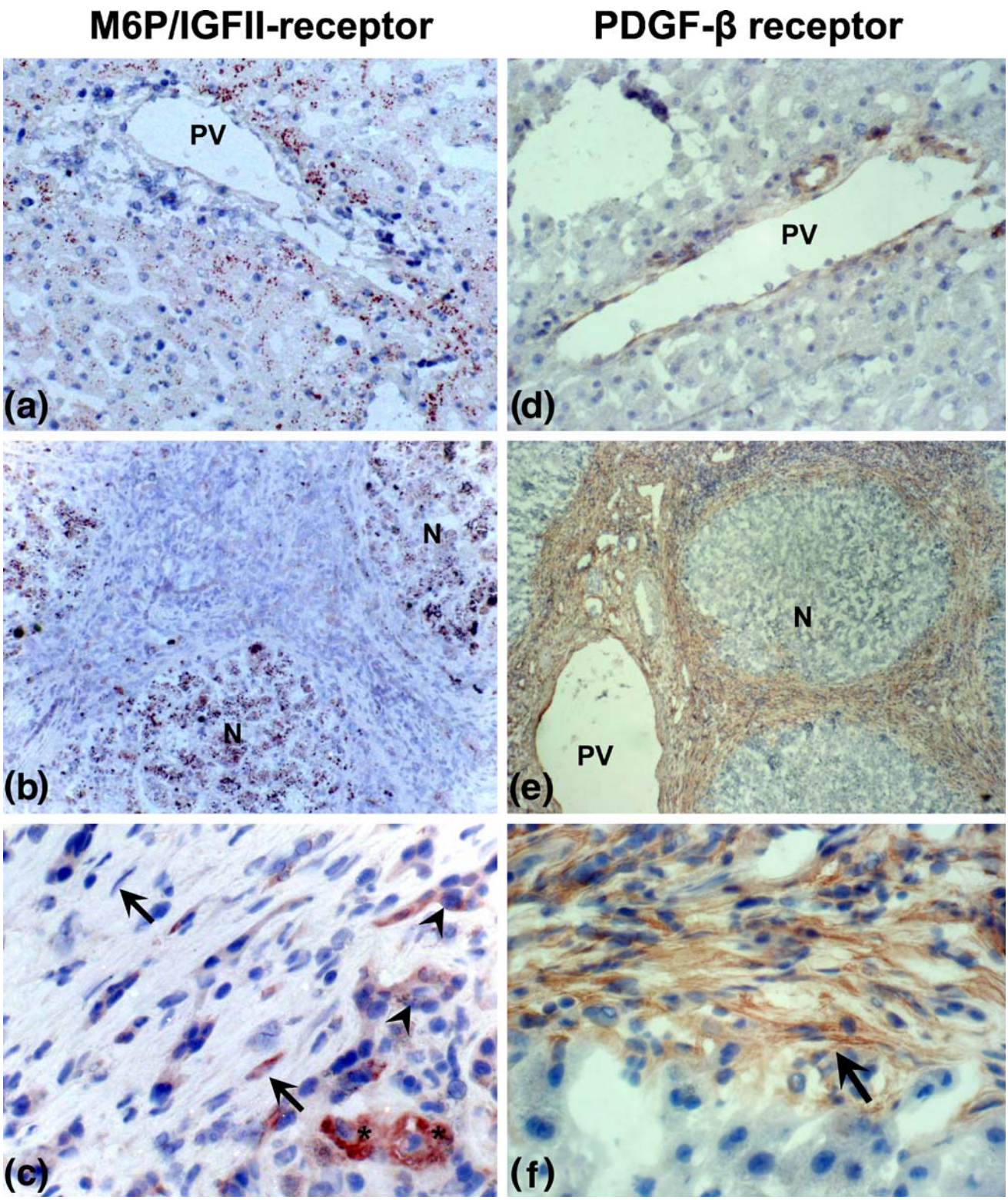

Fig. 5. Immunohistochemical detection of M6P/IGFII-receptor (a, b and c) and PDGF-β receptor (d, e and f) in healthy human livers (a, d; magnification 20 × 10) and cirrhotic human livers (b, magnification 10 × 10, C magnification 4 × 10). A high magnification (c, f; magnification 400×) shows that cells in the fibrous bands are positive for both receptors. An *asterix* indicates the localization of periseptal hepatocytes, the *arrow head* shows ductular cholangiocytes, and the *arrow* points to (myo)fibroblasts. *PV* = portal vein, *N* = cirrhotic nodule.

expression was still present in hepatocytes. However, additional staining for the receptor was found in cells in the fibrous bands, which are the areas within the liver characterized by excessive collagen deposition. The staining was predominantly detectable on cholangiocytes, both in cells lining the bile duct and bile ductular structures (Fig. 5c).

PDGF-β receptor expression in normal human livers was only found in the portal areas, in which the hepatic artery and portal vein were stained (Fig. 5d). Sinusoidal endothelial cells were not stained for the PDGF-receptor. The expression of the PDGF-β receptor was massively enhanced in cirrhotic human livers (Fig. 5e), and the receptor staining was mainly detectable in cells within the fibrous bands (Fig. 5f). Our observations are in line with the reported enhanced expression of PDGF-β receptor in activated myofibroblasts which are located in these areas (13).

DISCUSSION

In this study, we compared two HSC-specific carries for their ability to deliver $15dPGJ_2$ to fibrogenic cells in the liver. The first carrier M6PHSA, which is substituted with sugar groups as homing devices, binds to the M6P/IGFII receptor present on activated HSC (4). The second carrier pPBHSA, which is substituted with cyclic peptide homing devices, can bind to the PDGF-â receptor which is strongly increased on the cell-surface of activated HSC (5).

The interaction with HSC as reported for the individual carriers (5,7) was preserved after coupling of $15dPGJ_2$ to these carriers. The receptor-mediated binding of both 15dPGJ2 conjugates was inhibited by co-incubation with its own carrier, while no effect of HSA was found. These blocking experiments indicate that binding to these cells is mediated by specific receptors. In addition, co-incubation with polyinosinic acid, a known blocker for the scavenger receptor class A (29), strongly reduced the binding of $15dPGJ_2$-M6PHSA to the cells. The involvement of scavenger receptors was anticipated because these receptors bind negatively charged molecules and M6P-containing compounds have a net negative charge, as confirmed with FPLC-monoQ analysis. The expression of a scavenger receptor class B, CD36, was reported on HSC and a co-localisation with alpha-smooth muscle actin-positive HSC in human fibrotic livers was immunohistochemically demonstrated (30). However, inhibition by polyinosinic acid, as found in our studies, suggests that not scavenger receptor class B, but rather class A contributed to the accumulation of M6PHSA conjugates in the HSC. This scavenger receptor class A has not yet been described on HSC, but our results together with the observations presented by Adrian *et al.* (31) warrant further exploration of these receptors on HSC in rat and human livers. In contrast, binding of $15dPGJ_2$-pPBHSA to cultured HSC was not affected by polyinosinic acid, suggesting that scavenger receptors are not involved in the binding of pPBHSA to HSC. Moreover, preincubation with M6PHSA showed that $15dPGJ_2$-pPBHSA was not interacting with any of the binding domains of the M6P-carrier. This shows that both carries use different entry routes to deliver a drug within the HSC.

$15dPGJ_2$ was conjugated to the carrier via its carboxylic acid group (COOH). In contrast to the cyclopentenone ring of prostaglandins, the carboxylic acid group is not essential for the biological activity (32,33). In our studies, we showed that both conjugates were able to effectively reduce the viability of HSC *in vitro*. This implicates receptor-mediated endocytosis of the drug-carrier and intracellular degradation of the constructs in the lysosomes of the cell. Of note, the chemical amide bond between the carboxylic acid group of the prostaglandin and the lysine moieties within HSA cannot be cleaved by intracellular proteases as present in the lysosomes with part of the carrier attached to it. Using the same coupling procedures to couple naproxen to HSA, it was found that naproxen-lysine was released within the cell, after degradation of naproxen-HSA (34). Also, coupling of mycophenolic acid to HSA via this coupling procedure yielded not the parent compound (35). So, most likely $15dPGJ_2$ is also not released as the parent compound but despite this pharmacological activity is present.

Because of its apoptosis-inducing activities, $15dPGJ_2$ seems an attractive compound to apply in liver fibrosis (16–18). However, the therapeutic use of $15dPGJ_2$ may be limited because of high albumin binding (24) and expected poor pharmacokinetics as most of these lipophilic prostaglandins easily cross cell membranes. Also, our studies with HSC *in vitro* showed that serum completely blocked the activities of free $15dPGJ_2$, while both conjugated forms of $15dPGJ_2$ were still effective and reduced HSC viability almost comparable to serum-free conditions. The pharmacokinetic parameters of 15dPGJ2 after iv injection have not been reported, probably because so far no radioactive compound is available and HPLC detection is not sensitive enough. The obstacles as encountered in the therapeutic use of prostaglandins *in vivo* may be solved by targeting of the prostaglandin to the HSC by means of conjugation to HSC-specific carriers.

With regard to an expected low HSC accumulation of $15dPGJ_2$ *in vivo*, we now demonstrate a rather complete targeting to the fibrotic liver of both $15dPGJ_2$ conjugated forms. Respectively, 86 and 63% of the dose of $15dPGJ_2$-M6PHSA and $15dPGJ_2$-pPBHSA was present in the liver already 15 min after intravenous injection. Previously, we reported an hepatic accumulation of M6PHSA and pPBHSA of 59 and 48% of the administered dose, 10 min after iv injection (4,5). The higher liver accumulation as found in the present studies for $15dPGJ_2$ conjugated M6PHSA and pPBHSA may be caused by the attachment of the lipophilic compound prostaglandin. We also determined the intrahepatic localization of both HSC-targeted forms of $15dPGJ_2$. $15dPGJ_2$-M6PHSA predominantly accumulated in the HSC (64%) and Kupffer cells (39%). The high accumulation of the M6P-conjugate in Kupffer cells is probably related to the net negatively charged protein backbone of the construct which is recognized by scavenger receptors (both class A and B) on these Kupffer cells (29). The other construct $15dPGJ_2$-pPBHSA predominantly co-localizes with HSC and parenchymal cells. Hepatocyte uptake was relatively low, reflected by the diffuse staining and could therefore not be quantified. It is likely that the pPB-conjugate, as also proposed for the carrier pPBHSA (5), follows a similar distribution pattern as the growth factor PDGF-BB, which is taken up by hepatocytes after opsonization by α_2-macroglobulin (36).

Further studies to assess the effects of $15dPGJ_2$ in cells that take up the construct are required. Next to the uptake in

HSC, hepatocytes will take up pPBHSA-15dPGJ$_2$ whereas in case of the M6PHSA carrier, Kupffer cells and endothelial cells take up the product parallel to HSC. If effects are found within these cells, it will determine the choice of carrier. To date, no effects of 15dPGJ$_2$ have been reported in KC, EC or hepatocytes. To assess the effects in all cell types and the net effect on liver fibrosis, the constructs have to be administered in therapeutic amounts for a prolonged period of time. This is part of our future research program.

However, the situation within the human diseased liver has also to be taken into account and we decided to focus on this issue first and studied the expression of both receptors in human cirrhotic livers. We demonstrated expression of the M6P/IGFII receptors in hepatocytes of healthy livers, but in human cirrhotic livers an increased expression on fibrogenic cells was observed compared to normal livers. Expression of this receptor on fibroblasts in humans has been reported (37), however no data on hepatic stellate cells or myofibroblasts exist yet. We did find uptake of M6PHSA constructs in human myofibroblasts and liver slices *in vitro* (4), but no significant staining for M6P/IGFII receptor was found in these cells in the studied human livers. Maybe a detailed investigation of M6P/IGFII receptor expression during fibrogenesis in the human liver at various stages of disease and with various etiologies should be performed. The expression of M6P/IGFII receptors in hepatocytes as demonstrated in human livers was also noted in rats (38), but this hepatocyte receptor expression did not lead to any uptake of M6PHSA in this cell type (4,35,39). Only uptake of M6PHSA in non-parenchymal cells was found, probably the receptor expression in hepatocytes is mostly intracellularly, i.e., not within reach of extracellular plasma proteins.

The highly induced expression of the PDGF-β receptor in human cirrhotic livers as detected by us, correlated with its localization as reported by Pinzani *et al*. (13). The high expression of in particular the PDGF-β receptor compared to the expression of the M6P/IGFII receptor makes our carrier with the PDGF receptor-recognizing peptides, pPBHSA, the most attractive candidate for application in the human situation. Subsequent studies to examine the distribution of the drug carriers in human livers or patients will be necessary to examine the applicability of the carriers in the human situation, but at least both target receptors are present.

In summary, the coupling of 15dPGJ$_2$ to the HSC-specific drug carriers M6PHSA and pPBHSA clearly led to a rapid and near complete accumulation of the prostaglandin in the fibrotic liver with significant uptake in HSC. Both targeted forms of 15dPGJ$_2$ were pharmacologically effective *in vitro*, and coupling evidently improved the activity of this prostaglandin in the presence of serum. *In vitro* receptor interactions differed between both targeted constructs, which led to differences in the hepatocellular distribution *in vivo*. Studies on long-term effects and on the applicability in patients will be necessary to determine which construct is the most effective during disease. Nevertheless, in both cases, the HSC is the main target cell and in view of the apoptosis-inducing activities of this prostaglandin in HSC, these conjugates may be a valuable tool to attenuate the fibrogenic process within the liver.

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