Anti-fibrotic effects of 15d-Prostaglandin J₂ targeted to Hepatic Stellate Cells

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submitted
Liver fibrosis can occur in response to different forms of injury. During fibrosis, the hepatic stellate cell (HSC) becomes activated, proliferates and is the major producer of collagens. Regression of fibrosis is associated with apoptosis of HSC. 15-deoxy-Δ^{12,14}-Prostaglandin J₂ (15d-PGJ₂) has been shown to induce apoptosis, to inhibit proliferation and to reduce collagen synthesis in human myofibroblasts. The therapeutic use of this compound is however limited due to the lack of cell and tissue specificity and a very short half-life. However, selective delivery of 15d-PGJ₂ to the HSC may overcome this. The aim of this study, therefore, was to synthesize and study a 15d-PGJ₂-conjugate that selectively accumulates in the liver fibrogenic cells. 15d-PGJ₂ was chemically coupled to M6P-HSA, which is known to accumulate in HSC. In vitro assays with human hepatic myofibroblasts showed that this 15d-PGJ₂-M6P-HSA induced apoptosis and inhibited proliferation. The conjugate showed binding to isolated rat HSC and selective and rapid accumulation in the liver in vivo. After 15 minutes, 87% of the injected dose accumulated in the fibrotic liver. In vivo treatment with 15d-PGJ₂-M6P-HSA in BDL fibrotic rats revealed decreased procollagen 1α1 mRNA levels and a reduction in desmin positive cells. In conclusion, we developed the first cell-specific prostaglandin construct for HSC. This conjugate rapidly and almost completely accumulated in the liver and decreased in the number of HCS, associated with an attenuation of the synthesis of collagens within the fibrotic liver.
INTRODUCTION

Liver fibrosis is the common response to chronic liver injury, ultimately leading to cirrhosis and its complications, such as portal hypertension, liver failure and hepatocellular carcinoma. The fibrogenic process is consecutive to intense proliferation of myofibroblastic cells, that synthesize extracellular matrix components and inhibitors of matrix degradation\(^1,2\).

A potential therapy to treat liver fibrosis would be to inhibit the accumulation of fibrogenic cells (Hepatic Stellate Cells and myofibroblasts). In this respect, 15-deoxy-\(\Delta^{12,14}\)-Prostaglandin J\(_2\) (15d-PGJ\(_2\)) might represent an interesting drug. This cyclopentanone exhibits several effects including growth arrest, induction of apoptosis in different cell types and suppression of macrophage activation \(^3-5\). Recently, it was shown that this prostaglandin induced apoptosis of human hepatic myofibroblasts (hMF)\(^6\). Sub-apoptotic concentrations of 15d-PGJ\(_2\) strongly inhibited the proliferation of hMF and reduced the expression of interstitial collagens \textit{in vitro}\(^7,8\). 15d-PGJ\(_2\) is a downstream metabolite of cyclooxygenase and is derived from PGD\(_2\) by dehydration\(^9\). It was detected \textit{in vivo} at sites of inflammation during its resolution phase, suggesting that it might function as a negative feedback regulator of the inflammatory process\(^10\).

The therapeutic use of the lipophilic compound 15d-PGJ\(_2\) is limited by the lack of cell and tissue specificity, resulting in a low efficacy and a high risk of potential side effects. In general, all prostaglandins are locally acting mediators that are rapidly metabolized by the lung\(^11\) and cleared from the body by the kidneys resulting in a very short half life. For example, PGE\(_2\) (dinoprostone) has a plasma half-life (t\(_{1/2}\)) of less than 1 minute and PGI\(_2\) (epoprostenol) has a t\(_{1/2}\) of 2-3 minutes\(^12,13\). Also, the high protein binding of prostaglandins in serum\(^14\) prevents compounds such as 15d-PGJ\(_2\) to effectively reach the target cells within the liver after systemic administration.

Therefore, selective delivery of 15d-PGJ\(_2\) to HSC can be a promising strategy to get the active drug at the desired site. In recent years, carrier systems that allow selective delivery of drugs to HSC have become available. For instance, mannose-6-phosphate modified human-serum-albumin (M6P-HSA), which binds to the Insulin-like Growth Factor II / Mannose-6-Phosphate receptor (IGF-II/M6P), has been shown to accumulate rapidly in the HSC of fibrotic rats\(^15,16\).

The aim of this study therefore was to synthesize and test a 15d-PGJ\(_2\)-M6P-HSA conjugate that selectively affects the activated fibrogenic cells within the liver. The effects of this conjugate were determined \textit{in vitro} and \textit{in vivo} in rats with liver fibrosis.
MATERIALS AND METHODS

Materials

Human Serum Albumin (HSA) was purchased from the central laboratory of the blood transfusion services (Sanquin Blood Supplies, Amsterdam, The Netherlands). 15-deoxy-Δ\(^{12,14}\)-Prostaglandin J\(_2\) (15d-PGJ\(_2\)) was purchased from ITK Diagnostics (Uithoorn, The Netherlands). All other chemicals used were of analytical grade.

Animals

Male Wistar rats (Harlan, outbred strain, 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

HSA was modified with mannose 6-phosphate (M6P) groups and the products were purified and characterized according to standard procedures\(^{15,16}\). Coupling of 15d-PGJ\(_2\) to M6P-HSA: The carboxylic acid group of 15d-PGJ\(_2\) (10 mg dissolved in 150 µl dimethyl formamid) was activated with 11.7 mg N,N-dicyclohexylcarbodiimide (DCC, Sigma, St. Louis, MO, USA) dissolved in 80 µl dimethyl formamid and stirred for 1 hour at room temperature. Activated 15d-PGJ\(_2\) was subsequently added to 20 mg M6P-HSA (dissolved in PBS, 4 mg/ml) and stirred for 16 hours. This mixture was extensively dialyzed against milliQ water at 4°C to remove all low molecular weight compounds (including excess 15d-PGJ\(_2\)) and the conjugate was lyophilized and stored at -20°C.

The amount of 15d-PGJ\(_2\) coupled to the carrier was estimated by 15d-PGJ\(_2\) correlate-EIA kit (Assay Designs, Ann Arbor, Michigan, USA). In addition, high performance liquid chromatography (HPLC) methods were used to assess the amount of released 15d-PGJ\(_2\) after hydrolysis of the bond between and M6P-HSA by ascorbic acid (pH 2.5, overnight at 80°C). Samples were injected on a C18 Chromolith SpeedROD column (Merck KGaA, Darmstadt, Germany; Flow 1 ml/min, Acetonitril/H\(_2\)O/triflouroacetic acid: 50/50/0.1, detection 254 nm).

Human hepatic myofibroblast isolation

Human hepatic myofibroblasts (hMF) were obtained by outgrowth of explants of normal human livers\(^{17}\). This procedure was performed in accordance with ethical regulations imposed by French legislation. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA, USA), supplemented with 5% Fetal Calf Serum (FCS) and 5% Human Serum and used between the third and seventh passage. The myofibroblastic nature of the cells was routinely evaluated by microscopy and checked for the presence of α-Smooth Muscle Actin (α-SMA) by immunohistochemistry.

Rat hepatic stellate cell isolation

Primary HSC were isolated from livers of male Wistar rats (> 500 g, Harlan)\(^{18,19}\). The HSC were cultured in DMEM (Invitrogen) supplemented with 10% 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 the phenotype of activated HSC as assessed by microscopy and presence of α-SMA.
**Effects of liver targeted 15d-Prostaglandin J2**

**Experimental design: in vitro studies**

The biological activity of 15d-PGJ2 and 15d-PGJ2 coupled to M6P-HSA was assessed in hMFs by measuring proliferation and induction of apoptosis. Since *in vivo* experiments are performed in rats, biological effects and receptor binding of the construct were also analyzed in cultured rat HSC.

**Apoptosis assays on hMF**

Caspase-3-like activity was assayed on cell lysates according to standard methods. Briefly, after incubation of hMF (300,000 cells in 60-mm dishes) with 15d-PGJ2, carrier or conjugate, cells were lysed and DEVDase activity was measured in 200 µl of assay buffer, containing 50 µg of total protein and 20 µM AC-DEVDAFC (Biomol, Tebu, France) as fluorogenic substrate.

Nuclear morphology was measured with DAPI (Roche Diagnostics, Indianapolis, IN, USA) staining in cultures of non-confluent hMF incubated with 15d-PGJ2, carrier or conjugate according standard methods.

**Proliferation assay on hMF**

hMFs (10,000 cells/96-well) were allowed to attach overnight, serum-starved for 72 h and preincubated for 1 hour with the indicated compounds. Subsequently, human serum (5%) was added and 6 hours later, ³H-thymidine (0.5 µCi/well) was added and the cells were cultured for another 28 hours. Cells were washed and collected and radioactivity was measured with a β-counter.

**Biological effects on HSC.**

Protein binding assays in cultures of HSC were performed with ¹²⁵I-labelled 15d-PGJ2-M6P-HSA as described. Furthermore, viability of the HSC was assessed. HSC (5,000/96-well) were incubated for 24 hours in 200 µl medium with or without 10% FCS. Subsequently, 15d-PGJ2, 15d-PGJ2-M6P-HSA or M6P-HSA was added in increasing concentrations and the cells were incubated for another 18 hours. Alamar blue (20 µl, Serotec, Oxford, UK) was added and the cells were incubated for an additional 24 hours. The conversion of alamar blue redox indicator by metabolic activity of cells reflects the number of cells (i.e. net result of proliferation and apoptosis) present in each well. The alamar blue conversion was measured using a fluorimeter.

**Induction of fibrosis in vivo**

To induce liver fibrosis, rats (220-240 g, Harlan) were subjected to bile duct ligation (BDL). Ten days after ligation, (BDL-10d), rats were used for further experiments. At this point, liver fibrosis is evident but not excessive yet, while the IGF-II/M6P-receptor is upregulated.

**Organ distribution**

Organ distribution experiments were performed in BDL-10d rats. Animals were injected *i.v.* with a tracer dose of ¹²⁵I-15d-PGJ2-M6P-HSA and ¹²⁵I-HSA (1000,000 cpm per rat). Fifteen minutes after injection, the animals were sacrificed by heart puncture and organs were collected. Radioactivity in each organ was measured with a γ-counter and corrected for blood-derived radioactivity (n=3 per group).

**γ-camera imaging**

HSA and 15d-PGJ2-M6P-HSA were radiolabeled with ¹²³Iodine. Immediately after the *i.v.* injection of 5 MBq in the penile vein of BDL-10d rats, the distribution of the radiolabel was dynamically...
recorded using a γ-camera from 0 to 20 minutes with a frame rate of one scan per 30 seconds (n=3 per group).

**In vivo effect study**

To assess the effects of the compounds on the progression of liver fibrosis, we treated BDL-10d rats and examined these rats 24 hours later at day 11. Rats were *i.v.* injected with vehicle (PBS) or with M6P-HSA, 15d-PGJ$_2$-M6P-HSA (both 10 mg/kg) or 15d-PGJ$_2$ (400 µg/kg, equivalent to ten molecules of 15d-PGJ$_2$ coupled to one M6P-HSA molecule), all dissolved in PBS. After 24 hours, rats (n = 12 per group) were sacrificed and organs and blood were harvested and stored until further examination.

**Immunohistochemical stainings**

Acetone-fixed cryostat sections (4 µm) of liver slices were stained using indirect immunoperoxidase methods with peroxidase-conjugated secondary antibodies (DAKO, Glostrup, Denmark). HSC were detected with monoclonal antibodies against αSMA and desmin (both Sigma). Goat polyclonal antibodies against rat collagen type I and III (both Southern Biotech, Birmingham, USA) were used to stain collagen type I and III simultaneously in the livers. All stainings were analyzed with ImageJ software (National Institute of Health, Bethesda, Maryland, USA). On ten digital photos per liver (magnification of 100X), the total area stained positive for αSMA, desmin and collagen was measured and related to the total area analyzed.

**Real-time PCR**

Total RNA was isolated from rat livers using the RNeasy kit (Qiagen GmbH, Hilden, Germany) and the amount of RNA was measured with the NanoDrop ND1000 (Nanodrop Technologies, Wilmington, USA). The reverse transcriptase reaction (Promega, Madison, WI, USA) was performed with random primers. The transcription levels of rat collagen type Iα1 (forward, 5’- AGCCTGAGCCAGCAGATTGA-3’, reverse, 5’-CCAGGTTGACGCCTTTGTGA-3’), desmin (forward, 5’- AGGAACAGCGAGTCA GGTCTC-3’, reverse, 5’- AGAGCATCAATCTCGCAGGT-3’) and αSMA (forward, 5’- GACACCAGGGAGAT GATGTT-3’, reverse, 5’- GTTAGCAAGTGGTGTGCT-3’) were detected by quantitative real time PCR methods with SYBR Green on a ABI 7900HT apparatus (both Applied Biosystems, Foster City, CA, USA). Formation of single products was confirmed by analyzing the dissociation step at the end of each PCR reaction. The data were quantified via comparative ΔΔCt calculation (GAPDH as housekeeping), and the levels of gene expression in livers from normal rats were chosen as baseline.

**Statistics**

Results are expressed as mean ± SEM. The statistical analysis of the *in vitro* experiments were performed by a two-tailed Student’s t-test, the results of the *in vivo* effect studies were tested by a one-way ANOVA followed by the LSD post-hoc test. P values lower than 0.05 were considered statistically significant.
RESULTS

Characteristics of the conjugate 15d-PGJ$_2$-M6P-HSA

HSA was modified with activated aminophenyl-6-phospho-$\alpha$-D mannopyranoside to obtain M6P$_{33}$-HSA$^{15,16}$. The monomeric form of this product was separated from the dimeric and polymeric forms using preparative FPLC sephadex separation methods. Subsequently, 15d-PGJ$_2$ was covalently coupled to M6P-HSA to obtain the conjugate 15d-PGJ$_2$-M6P-HSA. The amount of 15d-PGJ$_2$ molecules coupled to each M6P-HSA protein was estimated to be 10:1 as assessed by HPLC and ELISA methods.

\[ \text{Figure 1: Apoptotic effects of 15d-PGJ}_2 \text{ and 15d-PGJ}_2\text{-M6P-HSA in cultures of human hMF. A: 15d-PGJ}_2 \text{ and 15d-PGJ}_2\text{-M6P-HSA induce caspase-3-activity in hMF in a concentration dependent manner. The estimated concentration of 15d-PGJ}_2 \text{ coupled to M6P-HSA is depicted as well as the protein concentration of 15d-PGJ}_2\text{-M6P-HSA. The results are expressed as the mean ± SEM (n=3, \( * = 0.05)). B: DAPI staining of the nuclei of hMF treated with 15d-PGJ}_2, 15d-PGJ}_2\text{-M6P-HSA, M6P-HSA or vehicle. Note the condensed nuclei in the 15d-PGJ}_2 \text{ and 15d-PGJ}_2\text{-M6P-HSA treated hMF (magnification 680X).} } \]

In vitro activity of 15d-PGJ$_2$-M6P-HSA on hMF

Previously, we showed that 15d-PGJ$_2$ induced apoptosis of human hepatic myofibroblasts$^3$. Incubation of hMF with 15d-PGJ$_2$-M6P-HSA also induced apoptosis in a concentration dependent manner. The highest concentration tested (50 µg/ml, corresponding with 6.4 µM 15d-PGJ$_2$) resulted in a strong induction of caspase 3 activity (25 ± 4 fold over control, Figure 1A). The carrier alone (M6P-HSA) did not
induce apoptosis of hMF, even at high concentrations (250 µg/ml, Figure 1A). DAPI staining of the nucleus confirmed the results found with the active caspase-3 assay (Figure 1B).

15d-PGJ₂ is also known to inhibit proliferation of hMF. In the present study, the incorporation of ³H-thymidine in hMF was significantly inhibited at a concentration of 2 µM 15d-PGJ₂ compared with control cultured hMF that were incubated with vehicle alone (Figure 2). Incubation of hMF with 15d-PGJ₂-M6P-HSA also resulted in inhibition of proliferation. ³H-Thymidine incorporation was significantly inhibited at a concentration when apoptosis was not detected yet (10 µg/ml, corresponding to 1.3 µM 15d-PGJ₂). A higher concentration of the conjugate resulted in a negative proliferation compared with control incubations, which is in agreement with the data presented in Figure 1A, showing induction of apoptosis at 25 µg/ml. Incubation of hMF with M6P-HSA alone had no significant effects on the proliferation compared with control incubations (Figure 2).

**Figure 2**: Effects of 15d-PGJ₂, 15d-PGJ₂-M6P-HSA or M6P-HSA on the proliferation of hMF as assessed by ³H-thymidine incorporation. Note that complete inhibition of proliferation can be achieved. The results are expressed as the mean ± SEM (n=3, * = p < 0.05).

**In vitro activity of 15d-PGJ₂-M6P-HSA on HSC**

To test whether 15d-PGJ₂-M6P-HSA selectively binds to receptors on HSC, we performed binding studies on culture-activated HSC with ¹²⁵I-labeled 15d-PGJ₂-M6P-HSA. In Figure 3 it can be seen that binding to HSC was significantly reduced by 81 ± 5% (P<0.05) after co-incubation with M6PHSA, which is an M6P/IGF-II receptor ligand. In contrast, preincubation with HSA only mildly affected the binding of ¹²⁵I-15d-PGJ₂-M6P-HSA to the HSC.

We also investigated the effects of 15d-PGJ₂ and 15d-PGJ₂-M6P-HSA on the viability in cultures of rat HSC and the influence of serum on this since albumin is known to affect the bioactivity of prostaglandins. The addition of 15d-PGJ₂ to cells
grown without FCS caused a significant and dose-dependent drop in viability of HSC (84 ± 17% reduction at 20 µM 15d-PGJ$_2$). In contrast, the same experiment in the presence of 10% FCS showed that serum abolished the effect of 15d-PGJ$_2$ on HSC (Figure 4A). Subsequently, we examined the effect of 15d-PGJ$_2$-M6P-HSA on HSC. Without FSC, a dose-dependent reduction in viability of HSC was measured starting at a concentration of 25 µg/ml 15d-PGJ$_2$-M6P-HSA, corresponding with 3.2 µM 15d-PGJ$_2$. In the presence of FCS, 15d-PGJ$_2$-M6P-HSA was still able to reduce the cell number, although the dose-response curve was shifted to the right (Figure 4B, 68 ± 11% inhibition at 250 µg/ml). M6P-HSA alone showed no effect at all on the cell number, either with or without FCS (Figure 4B).

**Figure 3:** Binding of $^{125}$I-15d-PGJ$_2$-M6P-HSA to activated HSC alone or with co-incubation with an excess of M6P-HSA or HSA (both 1 mg/ml). Note that M6P-HSA, a ligand for the IGF-II/M6P receptor strongly reduces cellular binding of the conjugate in contrast to HSA. The results are expressed as the mean ± SEM (n=3, * = p < 0.05).

**Figure 4:** Viability of isolated activated rat HSC incubated with different concentrations of **A:** 15d-PGJ$_2$, **B:** 15d-PGJ$_2$-M6P-HSA. The experiments are performed under serum starvation (closed bars) and in the presence of 10% FCS (open bars). M6P-HSA showed no effect on the viability of HSC. The control cells (incubated with vehicle) are set at 100% viability, whereas control wells without cells are set at 0% viability. The results are expressed as the mean ± SEM (n=3, * = p < 0.05)
**In vivo accumulation of 15d-PGJ$_2$-M6P-HSA in the fibrotic liver**

With a $\gamma$-camera, the biodistribution of $^{123}$I-HSA was compared with $^{123}$I-15d-PGJ$_2$-M6P-HSA in BDL-10d rats. The $\gamma$-camera allows visualization of the distribution profile and assessment of the distribution of the construct in time. Figure 5A shows the distribution of $^{123}$I-HSA in which radioactivity was seen in well-perfused organs like kidneys and in the heart/lung area throughout the observation period, reflecting the blood circulation. In contrast, experiments with $^{123}$I-15d-PGJ$_2$-M6P-HSA showed that within the first minute after injection already high levels of the conjugate were localized in the liver area. These levels were higher than the $^{123}$I-HSA levels and remained in the liver area until the end of the experiment (Figure 5B). From these data we choose 15 min after i.v. injection as a relevant time point to quantify the organ distribution of 15d-PGJ$_2$-M6P-HSA.

Figure 5: $\gamma$-Camera analysis of $^{123}$I-HSA and $^{123}$I-15d-PGJ$_2$-M6P-HSA

**A**: Whole body scan taken at the end of the 20 minute test period of the distribution of $^{123}$I-HSA (left) and $^{123}$I-15d-PGJ$_2$-M6P-HSA (right) in BDL-10d rats after iv injection. The intensity of radioactivity ranges from black (low intensity) to white (high intensity).

**B**: The dynamic curve of $^{123}$I-HSA (square) and $^{123}$I-15d-PGJ$_2$-M6P-HSA (triangle) uptake in the liver area in the first 20 minutes after i.v. injection. L = liver, B = brain, H = heart, K = kidney and I = injection site (n=3 per group).
Organ distribution revealed that fifteen minutes after i.v. administration, $^{125}$I-15d-PGJ$_2$-M6P-HSA accumulated significantly in the livers of BDL-10d rats (86 ± 7%), leaving very little conjugate in the blood (3.3 ± 0.2%). Less than 3% of the injected dose was taken up in other major organs and tissues including kidney, heart, lung and spleen (Figure 6). In contrast, organ distribution studies of $^{125}$I-HSA revealed that 57 ± 9% of the injected dose was still in the blood 15 minutes after injection (Figure 6). In conclusion, these pharmacokinetic studies show a rapid and almost complete targeting of the conjugate to the fibrotic liver.

![Figure 6: Quantitative analysis of in vivo distribution of $^{125}$I-labeled proteins, 15 minutes after i.v. injection in fibrotic rats (BDL-10d). Data show that 15d-PGJ$_2$-M6P-HSA accumulated in the fibrotic liver, whereas HSA remained in the blood (n=3 per group).](image)

**In vivo effects of 15d-PGJ$_2$-M6P-HSA.**

The pronounced effects of 15d-PGJ$_2$ and 15d-PGJ$_2$-M6P-HSA on HSC and hMF and the rapid accumulation of the targeted construct in fibrotic livers prompted us to treat fibrotic rats for 24h. We examined the effects of treatment on desmin, $\alpha$-SMA and interstitial collagens in these rat livers, 10 days after BDL, by immunohistochemical staining and mRNA expression levels.

Morphometric analysis of the livers stained for desmin showed a significant reduction in livers of the rats treated with 15d-PGJ$_2$ (39% reduction) and 15d-PGJ$_2$-M6P-HSA (35% reduction) compared with the PBS-treated rats (p<0.05). Treatment of rats with the carrier alone did not result in a difference compared with the PBS-treated rats. Single-dose treatment had also no effect on the amount of $\alpha$-SMA and collagen type I and III in these livers (Figure 7).

Figure 8 shows the effect of treatment on procollagen 1$\alpha$1 mRNA expression. The results are expressed as the fold induction over the expression level of procollagen1$\alpha$1 found in normal, non-fibrotic rat livers. Ten days after induction of fibrosis, a 33-fold increased collagen expression was found within the livers of the
PBS-treated rats. Treatment of rats with 15d-PGJ\textsubscript{2}-M6P-HSA resulted in a significant reduction in expression of procollagen1\textalpha1 mRNA levels compared with the PBS-treated groups (54% reduction). Rats treated with 15d-PGJ\textsubscript{2} and carrier alone showed a decline in procollagen 1\textalpha1 mRNA expression levels compared to the PBS-treated group but this was not significant. Treatment had also no significant effect on the mRNA expression levels of \textalpha-SMA and desmin found within these livers (Fig 8).

**Figure 7:** Results of the morphometric analysis of liver sections of the BDL-10d rats treated with different compounds stained for desmin, \textalpha-SMA and collagen type I and III. Hepatic desmin staining showed a significant decrease in the desmin positive area for rats treated with 15d-PGJ\textsubscript{2}-M6P-HSA or 15d-PGJ\textsubscript{2} compared to the PBS and M6P-HSA treated groups. Treatment did not affect the \textalpha-SMA and collagen type I and III staining 24 hours after a single injection (n=12 animals per group ± SEM, *p < 0.05. ANOVA).

**Figure 8:** Quantitative real-time PCR analysis of intrahepatic mRNA levels for desmin, \textalpha-SMA and procollagen 1\textalpha1 after treatment of BDL-10d rats with 15d-PGJ\textsubscript{2}, 15d-PGJ\textsubscript{2}-M6P-HSA, M6P-HSA alone or PBS. The procollagen 1\textalpha1 expression was significantly reduced when rats were treated with 15d-PGJ\textsubscript{2}-M6P-HSA compared to PBS-treated rats. The mRNA expression level of desmin and \textalpha-SMA were not significantly affected by the treatment. The mRNA expression was related to the mRNA levels of normal rats (n= 12 animals per group ± SEM, *p < 0.05. ANOVA).
DISCUSSION

Over the past few years, several reports have been published suggesting that 15d-PGJ$_2$ is an endogenous mediator during inflammation and has antifibrotic capacities $^3$-$^7$. Its actions include an attenuation of collagen synthesis, inhibition of proliferation and induction of apoptosis in HSC. 15d-PGJ$_2$ is, like all prostaglandins, a lipophilic compound that is produced locally at the site of inflammation. In general, systemic circulation of prostaglandins results in a rapid metabolization $^{11}$, excretion $^{12}$, and plasma protein binding $^{14}$, so they will only act locally. If 15d-PGJ$_2$ would be used as a therapy for liver fibrosis, high systemic concentrations must be administered to obtain sufficient effects within the liver. As a result, various adverse reactions can be anticipated when the distribution of this apoptosis-inducing agent is not restricted to the target area.

Targeting of prostaglandins to the desired site of action may solve these problems. In the past, prostacycline and prostaglandin E$_1$ have been encapsulated in liposomes to avoid their side effects and prolong their circulation time $^{24}$. These liposome-prostaglandin showed accumulation in the vascular lesion of hypersensitive rats $^{25}$. With this passive targeting approach, similar to pegylation of molecules, a prolonged circulation time leads to increased uptake in the target area. For targeting to HSC in fibrotic livers, this passive approach is not an option since passive hepatic uptake will occur most likely by hepatocytes and kupffer cells and not by HSC. Therefore, we designed a construct that actively targets the HSC by coupling 15d-PGJ$_2$ to a HSC-specific drug carrier. The M6P-HSA carrier binds specific to the IGF-II/M6P-receptor found on activated HSC $^{15,26}$. The free primary amine group (NH$_2$) of lysine groups in HSA allows coupling to the carboxylic group (COOH) of prostaglandins. In principal, every prostaglandin can be coupled via this reaction. After binding of M6P-containing ligands to the IGF-II/M6P-receptor, internalization of the ligand-receptor complex in endosomes occurs $^{16}$. In these endosomes, lysosomal enzymes degrade the M6P-containing ligands $^{27}$. For 15d-PGJ$_2$-M6P-HSA, this means a rapid internalisation and lysosomal breakdown of the conjugate resulting in the release of the prostaglandin intracellularly, probably coupled to an amino-acid since peptidases can not break down this non-peptide amide bond.

Despite this, the conjugate appeared to be biologically active. The conjugate induced apoptosis and inhibited proliferation of human and rat fibrogenic cells. Furthermore, the addition of serum did not abolish the effects of the conjugate, whereas serum completely blocked the effect of native 15d-PGJ$_2$, possibly by binding to the proteins in serum $^{14}$. This illustrates that the pharmacology and pharmaco-kinetics of 15d-PGJ$_2$-M6P-HSA is completely different compared with native 15d-PGJ$_2$. Assuming 10 molecules of 15d-PGJ$_2$ coupled to one M6P-HSA, the activity of the targeted compound in vitro was similar compared with the native 15d-PGJ$_2$. 
Coupling of 15d-PGJ$_2$ to M6P-HSA did not affect the selective binding of the drug carrier to its target receptor on HSC *in vitro*. Furthermore, *in vivo* γ-camera and distribution studies with radiolabeled 15d-PGJ$_2$-M6P-HSA showed a rapid and almost complete accumulation in the liver of BDL-10d rats, whereas HSA remained in the blood with almost no liver accumulation. These studies indicate that selective delivery of 15d-PGJ$_2$ to the fibrotic liver has been accomplished.

The *in vivo* effect of the conjugate was subsequently tested in 10d-BDL rats. After 10 days, liver fibrosis is evident but not excessive yet, while the IGF-II/M6P-receptor is already upregulated in these livers.$^{23}$ Twenty-four hours after a single injection, morphometric analysis of collagen-stained tissue showed no effects on the deposition of interstitial collagens in livers. Procollagen1α1 mRNA, however, showed a significant reduction (54%) in expression in animals treated with 15d-PGJ$_2$-M6P-HSA. This indicates that an initial step in the resolution of liver fibrosis, the reduction of collagen expression, has occurred within 24 hours after injection.

Morphometric analysis of the desmin stainings revealed a significant reduction of desmin-positive cells after treatment with 15d-PGJ$_2$ and 15d-PGJ$_2$-M6P-HSA. Untargeted 15d-PGJ$_2$ also caused an effect on the HSC, which was rather unexpected since the presence of serum resulted in a total blockade of 15d-PGJ$_2$ activity *in vitro*. Some reports, however, have also reported effects of 15d-PGJ$_2$ *in vivo* after IV administration.$^{4,28,29}$ Therapeutic dosages of 15d-PGJ$_2$ resulted in an attenuation of acute and chronic inflammation. Also anti-pyretic effects were reported with untargeted 15d-PGJ$_2$ in animal models.

Yet, 15d-PGJ$_2$-MP-HSA displayed a significant effect on procollagen 1α1 mRNA expression levels, whereas 15d-PGJ$_2$ did not. Systemic administration of untargeted 15d-PGJ$_2$ most likely results in relatively high peak concentrations, after which the concentration quickly drops by removal of the lipophilic compound. This might be just sufficient to affect the desmin-positive cells, but the transient effect on mRNA levels is gone after 24 hours. The conjugate, however, is slowly degraded in the lysosomes, resulting in sustained release of 15d-PGJ$_2$ from its carrier. The receptor-mediated endocytosis and the lysosomal degradation are rate-limiting steps which probably results in prolonged effects effect on procollagen 1α1 mRNA expression levels that are still detectable 24 hours after injection.

In summary, we developed the first HSC-specific prostaglandin construct. This conjugate showed clear biological effects in rat HSC as well as in hMF *in vitro* and a rapid and almost complete accumulation was found in the fibrotic rat liver. The conjugate attenuated the synthesis of collagen 1 *in vivo* in fibrotic rats after a single injection and decreased the number of desmin-positive cells within the fibrotic liver. Thus, direct effects on key parameters of fibrosis are demonstrated with this targeted 15d-PGJ$_2$ approach.
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REFERENCE LIST
