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The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue

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Abstract

Platelet derived growth factor (PDGF) is a key factor in the induction and progression of fibrotic diseases with the activated fibroblast as its target cell. Drug targeting to the PDGF-receptor is explored as a new approach to treat this disease. Therefore, we constructed a macromolecule with affinity for the PDGF-β receptor by modification of albumin with a small peptide that recognises this PDGF-β receptor. The binding of the peptide-modified albumin (pPB-HSA) to the PDGF-β receptor was confirmed in competition studies with PDGF-BB using NIH/3T3-fibroblasts and activated hepatic stellate cells. Furthermore, pPB-HSA was able to reduce PDGF-BB-induced fibroblast proliferation *in vitro*, and proved to be devoid of proliferation-inducing activity itself. We assessed the distribution of pPB-HSA *in vivo* in two models of fibrosis and related the distribution of pPB-HSA to PDGF-β receptor density. In rats with liver fibrosis (bile duct ligation model), pPB-HSA quickly accumulated in the liver in contrast to unmodified HSA ($P < 0.001$). The major part of pPB-HSA in the fibrotic liver was localized in hepatic stellate cells. In rats with renal fibrosis (anti-Thy1.1 model), pPB-HSA also homed to the cells that expressed the PDGF-β receptor, i.e. the mesangial cells in the glomeruli of the kidney. These results indicate that pPB-HSA may be applied as a macromolecular drug-carrier that accumulates specifically in cells expressing the PDGF-β receptor, thus allowing a selective delivery of anti-fibrotic agents to these cells.

Keywords: PDGF; Fibroblast; Liver fibrosis; Hepatic stellate cells (HSC); Glomerulosclerosis; Drug targeting

1. Introduction

Fibrotic diseases can occur in many organs of the body and in all cases this disease is associated with a gradual loss of tissue architecture and normal cell activities, leading to end-stage organ dysfunction [1]. In general, no pharmacotherapeutical intervention is available to treat fibrotic processes, which warrants the search for effective anti-fibrotic drugs. The main features of fibrosis are proliferation of fibroblast-like cells and excessive deposition of extracellular matrix products. The application of many anti-fibrotic drugs is hampered by the fact that they do not accumulate sufficiently into the target cells or cause to many side effects elsewhere in the body. In particular fibrosis, which is part of the normal tissue rearrangement process in all organs, calls for a cell-specific delivery of drugs.

A prominent stimulator of fibroblast proliferation during fibrosis is PDGF [1]. PDGF is a dimeric molecule composed of A and/or B chains. The PDGF isoforms, -AA, -AB, or -BB, exert their effects on target cells by binding to two structurally different receptors. The PDGF-receptor α binds to both A- and B-chains with high affinity, whereas PDGF-receptor β only binds the B-chain [2–4]. Recently, two other isoforms were identified, i.e. PDGF-C and PDGF-D [5–8]. Both forms require proteolytic activation before binding to and activation of, respectively, PDGF-α or -β receptors.

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Abbreviations: PDGF, platelet derived growth factor; HSA, human serum albumin; pPB-HSA, albumin modified with PDGF receptor-recognizing peptides (C*SRNLIDC*); HSC, hepatic stellate cells; BDL3, 3 weeks after bile duct ligation.

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In liver fibrosis, PDGF is the most important mediator involved in the proliferation and activation of HSC, which transform subsequently into myofibroblasts. The HSC, being the major producers of extracellular matrix constituents, are crucial in the pathogenesis of liver fibrosis [9]. The local production of PDGF is increased during fibrosis, and the *de novo* expression of the PDGF-β receptor is strongly enhanced on activated HSC [10–12]. Similarly, proliferation of fibroblast-like cells also occurs in renal fibrosis (glomerulosclerosis or tubulointerstitial fibrosis). Again, the enhanced expression of PDGF receptors on the fibroblast-like cells (mesangial cells and interstitial fibroblasts) as well as an increased production of PDGF plays a key role in the development of fibrotic processes in the kidney [13–16]. Therefore, HSC and mesangial cells are the major target cells for anti-fibrotic therapies in the liver and in the kidney, respectively.

The general event of the induction of PDGF-β receptors on cell membranes of fibroblasts after the onset of fibrosis prompted us to examine the possibility of developing a drug carrier that distributes to this receptor in order to obtain homing to the target cells in fibrotic tissues. This strategy finally aims at an improvement of pharmacological effects of drugs to treat fibrotic and sclerotic diseases and simultaneously decrease adverse effects of the chronically applied drugs. To create a drug carrier that specifically distributes to fibrogenic cells, we coupled a PDGF receptor-recognizing peptide to albumin. This novel peptide (C*SRNLIDC*) contains the amino acids arginine (R) and isoleucine (I), as being the receptor-binding moieties of the PDGF B-chain [17], together with the amino acids flanking these moieties in the native growth factor. Cyclization of the peptide, obtained by cys–cys interaction, was employed based on observations that cyclic peptides display stronger binding to their receptor than the linear analogues [18,19]. The use of small peptides as specific receptor ligands has been described amongst others for targeting to integrin receptors [18,20,21]. This study is the first to describe a PDGF receptor-recognizing peptide, and its application for targeting purposes. We studied the binding of albumin modified with these PDGF receptor-recognizing peptides (pPB-HSA) to the PDGF receptor on fibroblasts *in vitro*, and *in vivo* in a rat model of liver fibrosis as well as in a model of renal fibrosis.

2. Materials and methods

2.1. Synthesis and characterization of pPB-HSA

The cyclic peptide C*SRNLIDC* was prepared by Ansynth Service BV and covalently coupled to HSA (obtained from Sanquin, Central Laboratory of Blood Transfusion Services), as described previously [21]. pPB-HSA was characterized with polyacrylamide gel electrophoresis (7.5%), fast protein liquid chromatography (Superdex-200 column (Pharmacia)), and circular dichroism [21].

2.1.1. Radiolabeling

pPB-HSA and HSA were labeled with ^{125}I or ^{123}I (gamma camera studies) according to the NBS method [22]. PDGF-BB (SanverTech) was labeled with ^{125}I according to the Bolton and Hunter method [23]. Prior to each experiment, the percentage of uncoupled radioactivity in the preparations was determined after precipitation of the protein-bound radioactivity with 10% trichloroacetic acid. Uncoupled radioactivity was removed by dialysis against phosphate-buffered saline (PBS) until the preparation contained <5% free iodine.

2.2. Binding of pPB-HSA to PDGF receptors

The binding of pPB-HSA to PDGF receptors was examined as described by Engström *et al.* [24]. Briefly, NIH/3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamin, 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). Confluent cultures in 12-well plates were washed with ice-cold binding buffer (PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgSO_4$, and 1 mg/mL BSA). Cells were incubated for 90 min with different concentrations of pPB-HSA (0–25.5 nmol/mL), cyclic peptide C*SRNLIDC* (0–340 nmol/mL), or HSA (0–300 nmol/mL) at 4°. Higher concentrations of pPB-HSA (>2 mg/mL) yielded an opalescent solution reflecting poor solubility of the modified protein at these concentrations. Subsequently, ^{125}I-PDGF-BB (50,000 cpm/well) was added and the cells were incubated for another 60 min. After washing of the cells with cold binding buffer (5×), they were lysed at room temperature in lysis buffer (20 mM Tris–HCl, pH 7.5 containing 1% Triton X-100 and 10% glycerol) and the radioactivity was determined with a γ-counter. The binding of ^{125}I-PDGF-BB in the presence of the various compounds was compared to the cellular binding of ^{125}I-PDGF-BB alone. The curves were fitted using a non-linear regression fit with the computer program GraphPad Prism.

2.3. Proliferation assay

2.3.1. Direct effect of pPB-HSA on cell proliferation

NIH/3T3 fibroblasts were seeded in plastic 96-well plates to a density of 10,000 cells/well. After 4 hr, the cells were cultured under serum-free conditions for the remainder of the experiment. 48 hr after seeding, PDGF-BB (150 ng/mL), pPB-HSA or HSA (both 29.4–29,400 ng/mL) were added to the wells and the cells were incubated at 37° for another 72 hr. [^3H]-Thymidine incorporation was measured during the last 18 hr of the total incubation period. Subsequently, the cells were washed and harvested to count the total incorporated cellular radioactivity.

2.3.2. Effects of pPB-HSA on PDGF-induced cell proliferation

For these particular studies, pPB-HSA or HSA (29.4–2940 ng/mL) were added to the cells 15 min before the addition of 150 ng/mL PDGF-BB at 48 hr after cell seeding. The rest of the experiment was performed identically as described in the previous section.

2.4. HSC binding experiments

Rat HSC were isolated and purified using Nycodenz according to standard techniques. After the isolation, the cells were cultured in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin (cDMEM). Cells cultured for 2 days exhibited the phenotype of quiescent HSC, while cells cultured for 10 days showed signs of the activated phenotype (α-smooth muscle positive and loss of vitamin A-lipid droplets).

The cellular binding and uptake of ^{125}I-pPB-HSA (50,000 cpm) was assessed during a 2-hr incubation period, as described [21,25], and compared to ^{125}I-HSA. The specificity of ^{125}I-pPB-HSA binding to HSC at 4° was evaluated after simultaneous incubation of ^{125}I-pPB-HSA with PDGF-BB and PDGF-AA (40 nM), and also with the PDGF receptor-blocking agents suramin (200 μM, obtained from ICN Biomedicals) and trapidil (4 mM, obtained from ICN Biomedicals) [26,27]. Unlabeled pPB-HSA (30 μM) was used as a control substrate to block the binding of ^{125}I-pPB-HSA.

2.5. Animals

Male Wistar rats (Harlan) were housed under standard laboratory conditions and had free access to food and water. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals and was performed according to strict governmental and international guidelines on animal experimentation.

2.5.1. Model of liver fibrosis

The rats were submitted to common bile duct ligation (BDL), and used for *in vivo* experiments 3 weeks after BDL [21]. Previous studies had shown that in the livers of these rats abundant HSC proliferation and activation, as well as excessive matrix deposition could be detected.

2.5.2. Model of kidney fibrosis

A single dose of 5 mg/kg anti-Thy1.1 IgG (obtained from Department of Pathology, Groningen, The Netherlands) was i.v. injected in rats to induce glomerulosclerosis [28,29]. At 21 days after injection, the rats were used for *in vivo* experiments. At this time point, the kidneys displayed various signs of fibrosis, that is mesangial cell activation and proliferation, and deposition of extracellular matrix in the glomeruli.

2.6. In vivo experiments

2.6.1. Gamma camera studies

Anaesthetized rats were placed on a low-energy collimator of a gamma camera, and ±1.5 MBq ^{123}I-labeled pPB-HSA or HSA was i.v. injected via the penile vein. The radioactivity was recorded for 30 min with a resolution of 1 min/time frame [30].

2.6.2. Organ distribution studies with radioactive detection

The *in vivo* distribution of ^{125}I-pPB-HSA, ^{125}I-HSA, and ^{125}I-PDGF-BB (tracer dosis: ±800,000 cpm per rat) were assessed at 10 min after i.v. administration via the penile vein [21].

2.6.3. In vivo localization with immunohistochemical detection

At 10 or 30 min after i.v. injection of 10 mg/kg pPB-HSA of HSA via the penile vein, the liver, kidneys (perfused thoroughly with ±10 mL saline), spleen, and lungs were removed and frozen in isopentane (−80°). Cryostat sections (4 μm) were immunohistochemically stained for the presence of pPB-HSA with a rabbit polyclonal antibody directed against HSA (ICN Biomedicals GmbH). The PDGF-β-receptor was detected with the rabbit polyclonal antibody PDGFR-β (958; Santa Cruz Biotechnology). Double-immunostaining techniques were used to examine the cellular localization of pPB-HSA as described [21]. In the liver, HSC were identified by a combination of two mouse monoclonal primary antibodies: desmin (ICN Pharmaceuticals) and glial fibrillary acidic protein (GFAP; Biogenex). The parenchymal cells, Kupffer cells and endothelial cells were identified, respectively, with the antibodies NTCP (Na^+/taurocholate cotransporting polypeptide present in the hepatocyte plasma membrane, kindly provided by B. Stieger, Zurich, Switzerland), ED2, and HIS52 (both Serotec). In the kidney, the activated mesangial cells were identified by α-smooth muscle actin (Sigma Chemical Co.).

2.6.4. Immunohistochemical analysis

The difference in pPB-HSA staining between the parenchymal and non-parenchymal cells was measured quantitatively using Leica Qwin image analysis software (Leica Microsystems B.V.). The area (μm^2) positively stained for pPB-HSA in parenchymal or in non-parenchymal cells was measured and related to the total area scanned (μm^2). These morphometric measurements were performed at a magnification of 800×, which allowed cell-specific measurements.

The sections double-stained for the modified albumin and the cell markers were quantitatively evaluated as described [21]. The number of double-positive cells was related to the total number of HSA-positive cells yielding the relative binding of pPB-HSA to each cell type.

2.7. Statistical analysis

Data was statistically analyzed with an unpaired Student's *t*-test and differences were considered significant at $P < 0.05$.

3. Results

3.1. Characterization of pPB-HSA

The cyclic peptides C*SRNLIDC* were prepared and coupled to HSA. The molecular weight of pPB-HSA was increased as compared to HSA, as detected with polyacryl amide gel electrophoresis. The increase in molecular weight indicated that pPB-HSA consisted of HSA substituted with a mean of 15 peptide groups. Additionally, electrophoretic and chromatographic analysis revealed that the major part of the pPB-HSA preparation consisted of monomeric protein. Furthermore, using circular dichroism analysis, we did not observe significant differences in the spectrum obtained with pPB-HSA as compared to unmodified HSA, indicating that the amount of α-helices and β-sheets in pPB-HSA was similar to HSA. This led to the conclusion that major conformational changes in HSA were not induced after chemical attachment of the peptide moieties. Similar results were previously obtained after modification of albumin with another cyclic peptide (C*GRGDSPC*) [21]. Polymerization of proteins or changes of in the structure are two important features with regard to potential immunogenicity and prevention of phagocytosis by the reticulo-endothelial system [31–33].

3.2. Binding of pPB-HSA to PDGF receptors in vitro

PDGF-BB and pPB-HSA displayed concentration-dependent binding to NIH/3T3 fibroblasts, in contrast to ^{125}I-HSA which did not bind to these cells (data not shown). To determine whether pPB-HSA specifically binds to PDGF receptors, we incubated the 3T3 fibroblasts with ^{125}I-PDGF-BB in the presence of increasing concentrations of pPB-HSA, HSA, or PDGF-peptide (pPB) (Fig. 1). We found that concentrations of pPB-HSA ≥1 nmol/mL decreased the cellular binding of ^{125}I-PDGF-BB. This receptor competition was not observed when HSA or the PDGF-peptide alone (pPB) was co-incubated with ^{125}I-PDGF-BB, not even at >10 times higher concentrations (up to 300 μM).

3.3. Effects of pPB-HSA on PDGF-induced cell proliferation

The growth factor PDGF-BB stimulated the proliferation of NIH/3T3-fibroblasts as measured by [^{3}H]-thymidine incorporation (Fig. 2A). The modified albumin pPB-HSA itself did not enhance [^{3}H]-thymidine incorporation, either

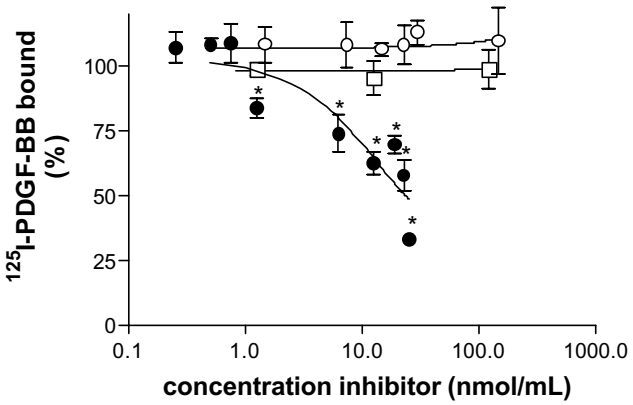

Fig. 1. Binding of PDGF-BB (^{125}I-labeled) to the PDGF receptors in confluent cultures of NIH/3T3 fibroblasts in the presence of pPB-HSA (●: 0–25 μM), HSA (○: 0–150 μM), or cyclic peptide pPB (□: 0–125 μM). Of note, only in the presence of pPB-HSA, binding of ^{125}I-PDGF-BB to the cells is inhibited ($^*P < 0.05$). Results are expressed as the mean ± SEM (N = 3).

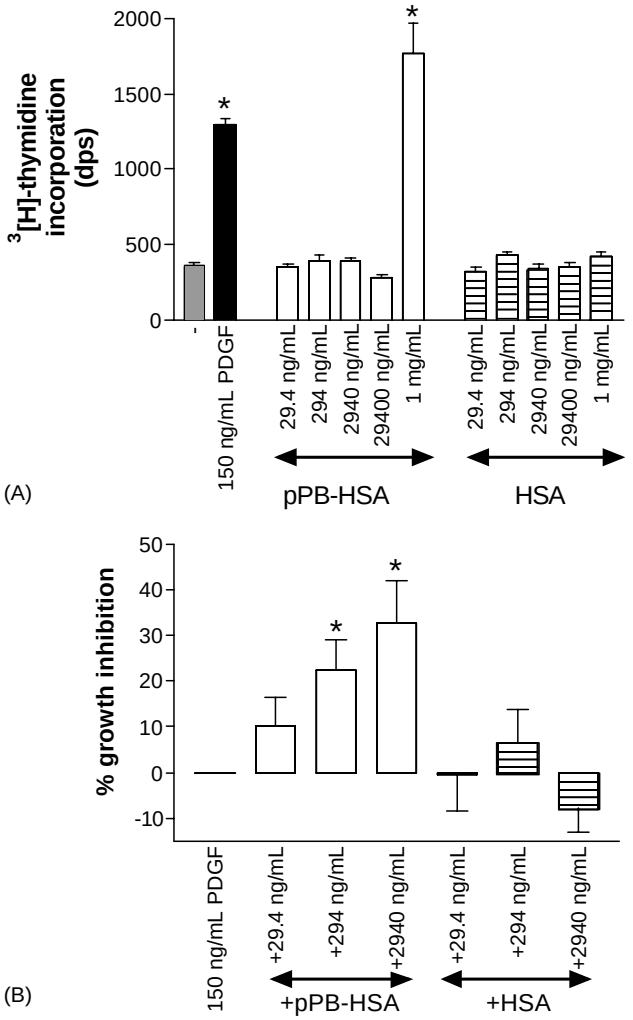

Fig. 2. (A) The effect of PDGF-BB (closed bar), pPB-HSA (open bars) or HSA (striped bars) on [^{3}H]-thymidine incorporation by 3T3 fibroblast cultures. (B) Dose–response relationships of pPB-HSA (open bars) and HSA (striped bars) on PDGF-induced 3T3-cell proliferation. Results are expressed as the mean ± SEM (N = 4, $^*P < 0.01$).

when added in an equimolar concentration as compared to PDGF-BB (i.e. 29.4 ng/mL), or when added in a 10–1000 M excess. Only very high concentrations of pPB-HSA (1 mg/mL) enhanced the [^{3}H]-thymidine incorporation. Changes in [^{3}H]-thymidine incorporation in the fibroblast cultures were never observed when unmodified HSA was added to the cells.

The effects of the modified albumin pPB-HSA on fibroblast proliferation induced by PDGF-BB were studied (Fig. 2B). After stimulation of the cells with 150 ng/mL PDGF-BB, a dose-dependent reduction in cell proliferation was seen when pPB-HSA was added ($P < 0.01$). Similar concentrations of unmodified HSA did not interfere with the PDGF-induced cell proliferation.

3.4. pPB-HSA and liver fibrosis

The expression of the PDGF-β receptor is enhanced in liver fibrosis, in particular on cell membranes of activated HSC [11,12]. Whereas staining for the PDGF-β-receptor in normal rat livers was absent, an increase in staining was found in rat livers up to 3 weeks after ligation. The staining was predominantly found in cells around the bile ducts, i.e. myofibroblasts or portal fibroblasts (Fig. 4B). BDL3-rats were used to examine the distribution of pPB-HSA. Imaging studies with a gamma camera demonstrated that already at 2–3 min after i.v. injection, pPB-HSA (^{123}I-labeled) was found in the liver region (Fig. 3A). The hepatic radioactivity increased in time and 6–7 min after administration the radioactivity was predominantly seen in the liver. HSA itself displayed a more general distribution pattern during the time-period measured; while the area with radioactivity was larger, the γ-radiation intensity per area was less as compared to pPB-HSA. In fact, ^{123}I-HSA was found throughout the body, but most clearly in the heart, lung, and liver region. This is most likely to be related to the high blood content of these organs. The hepatic content remained the same after the initial distribution phase that lasted for 2 min after injection, and was significantly lower than for pPB-HSA.

To quantify the liver selectivity of the pPB-HSA construct in more detail, direct tissue measurements with ^{125}I-labeled proteins were performed at 10 min after i.v. administration. These studies also revealed that ^{125}I-pPB-HSA was mainly taken up by the liver; 48 ± 9% of the total injected radioactive dose was detected in the liver at $t = 10$ min (Fig. 4A). At this time point 34 ± 2% of the radioactivity was still present in the blood compartment. Some extrahepatic accumulation of radioactivity was found in kidney, spleen, and lung. However, together these organs contained less than 10% of the initial dose. In contrast, ^{125}I-HSA was completely traced back in the blood at 10 min after injection, no uptake was found in the liver [21]. The plasma clearance of pPB-HSA was much slower than of PDGF-BB itself, of which only 1.6 ± 0.3% of the dose was detectable in blood at 10 min after injection. Also, radiolabeled PDGF-BB distributed mainly to the liver (70 ± 8%), while additional distribution occurred to kidney, spleen and lung (in total 18 ± 4%).

Ten min after injection of 10 mg/kg pPB-HSA, we confirmed immunohistochemically that the liver displayed the highest staining for pPB-HSA, whereas only a faint staining for pPB-HSA was detectable in the spleen, kidneys and lungs of BDL3 rats. Rats receiving unmodified HSA exhibited only staining in the vascular lumen of the liver or any other organ. The immunohistochemical results,

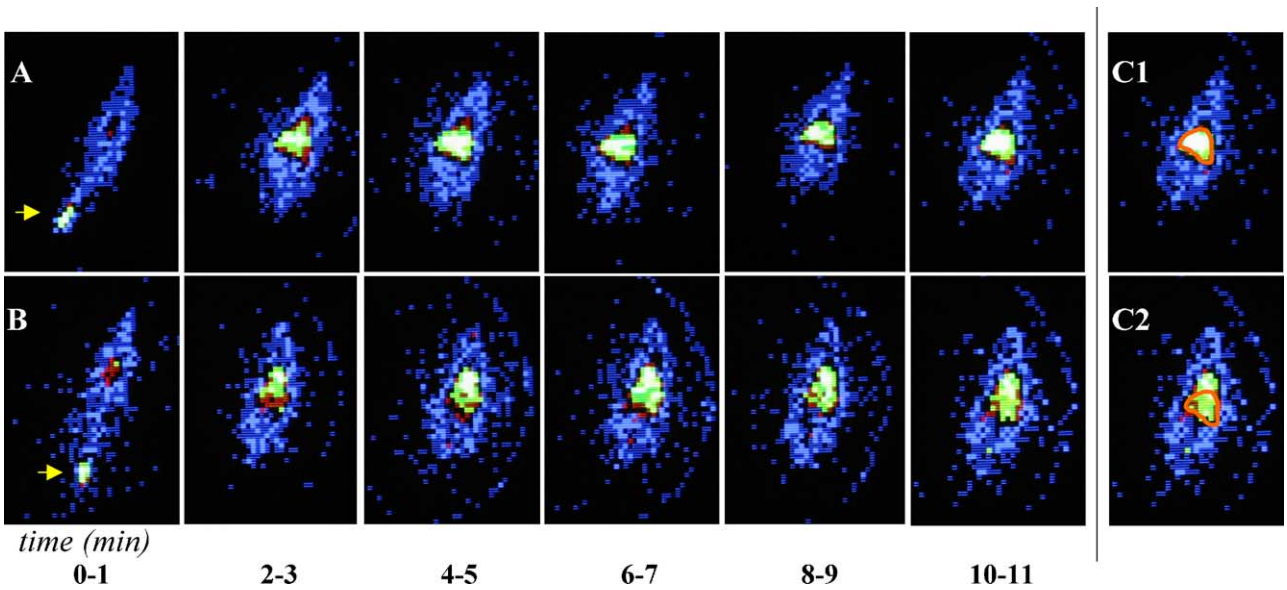

Fig. 3. Representative example of the distribution patterns of pPB-HSA (A) and HSA (B), both ^{123}I-labeled, after i.v. injection (arrow) via the penile vein in rats with liver fibrosis (BDL3), as determined using a gamma camera (N = 3). The intensity of the radioactivity per area is indicated by a color varying from dark blue (low intensity) to white (maximum intensity). Note the rapid uptake of pPB-HSA in the liver, already after 2–3 min, whereas the major part of HSA remains in the heart-lung region. (C) The liver region (purple) is depicted in the image of pPB-HSA (C1) and HSA (C2) obtained at 10 min after injection.

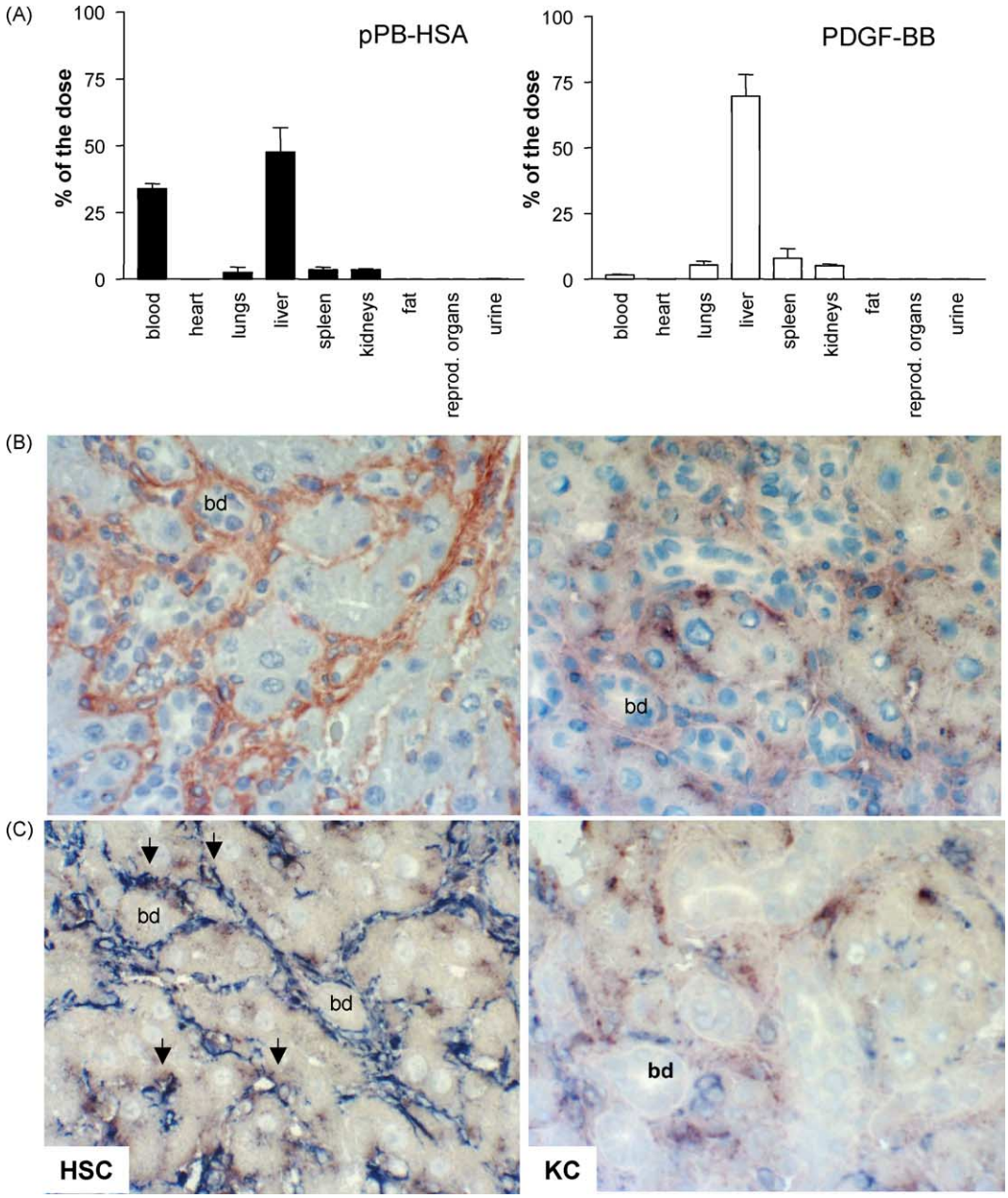

Fig. 4. Distribution of pPB-HSA in rats with liver fibrosis (BDL3). (A) Organ distribution ^{125}I-PDGF-BB and ^{125}I-pPB-HSA at 10 min after i.v. administration. The results are expressed as the mean ± SD ($N \geq 3$). (B) Immunohistochemical demonstration of PDGF-β receptor (left) and pPB-HSA (right) in livers at 3 weeks after ligation. (C) Co-localization of pPB-HSA (red staining) and HSC (stained with desmin/GFAP antibodies in blue) as obtained after double staining of the sections. Double positive cells were found (arrows) demonstrating uptake by HSC. In contrast, pPB-HSA staining did not correlate with staining for the KC marker ED2 (blue staining); bd: bile ducts. Original magnification: 400×.

obtained with unlabeled proteins, supported and extended the radioactive studies. Within the fibrotic liver, pPB-HSA was shown to be localized in non-parenchymal cells and in hepatocytes. The difference in the pPB-HSA staining between parenchymal and non-parenchymal cells was assessed with microscopical image analyzing techniques. The staining for pPB-HSA per area was $6 \pm 2\%$ for parenchymal cells and $37 \pm 14\%$ for non-parenchymal cells in the BDL3 livers. In addition to the higher staining area per cell, staining intensity for non-parenchymal cells was invariably higher than for the parenchymal cells, leading to the conclusion that accumulation of pPB-HSA predominantly occurred non-parenchymal cells.

Further staining for pPB-HSA in livers of BDL3 rats to identify the cells that take up the protein, showed similarities between the localization of the PDGF-β-receptor (Fig. 4B) and pPB-HSA staining. After double-staining of the liver sections, co-localization of pPB-HSA was found with the HSC-markers desmin and GFAP (Fig. 4C). A few double positive endothelial and Kupffer cells were found. Quantitative evaluation showed that $67 \pm 18\%$ of the double-positive non-parenchymal cells

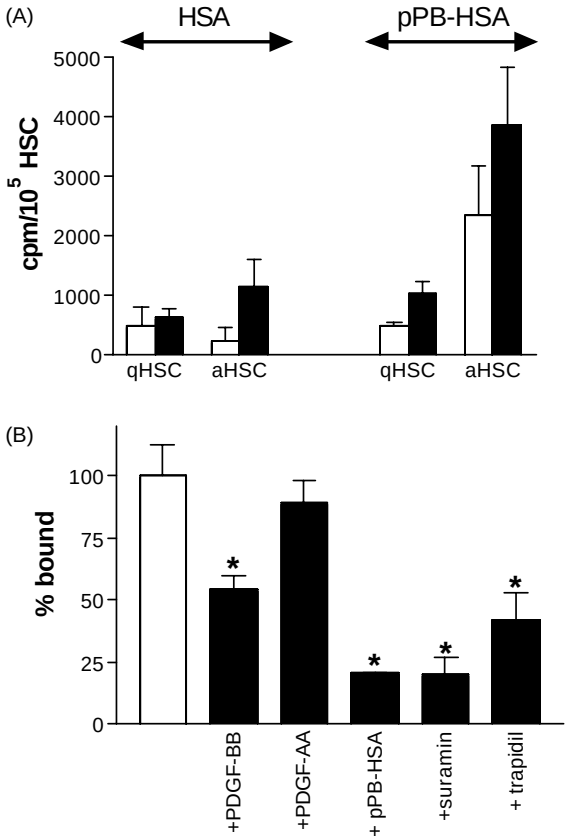

Fig. 5. (A) *In vitro* binding of ^{125}I-pPB-HSA and ^{125}I-HSA to cultures of quiescent HSC (qHSC) and activated HSC (aHSC). Cells were incubated with the compounds for 2 hr at 4° (open bars) and 37° (closed bars). Note the difference between ^{125}I-pPB-HSA and ^{125}I-HSA binding in cultures of activated HSC. No significant differences were found between 4 and 37°. (B) Binding of ^{125}I-pPB-HSA to activated HSC (at 4°) in the presence of the endogenous growth factors PDGF-BB and PDGF-AA (both 4 nM), unlabeled pPB-HSA (30 μM), suramin (200 μM), or trapidil (4 mM). Results are expressed as the mean ± SEM (N = 3, $^{*}P < 0.05$).

were identified as HSC, and respectively 14 ± 7 and 14 ± 4% could be identified as Kupffer and endothelial cells.

The binding of pPB-HSA to HSC *in vivo* was confirmed *in vitro* with primary cultures of quiescent and activated HSC (Fig. 5). After 2 hr of incubation, only the activated HSC significantly bound pPB-HSA as compared to HSA (Fig. 5A). The cell-bound ^{125}I-pPB-HSA at 37° was not significantly higher than at 4°, which indicated that no internalization occurred. The specificity of the binding of pPB-HSA to activated HSC was examined (Fig. 5B), and it was shown that the amount of cell-bound radioactivity was decreased by 80% in the presence of an excess of unlabeled pPB-HSA. In the presence of the PDGF receptor antagonists suramin (200 μM) and trapidil (4 mM), the binding of ^{125}I-pPB-HSA to HSC declined to, respectively, 20 and 42% of the total binding. Moreover, the binding of ^{125}I-pPB-HSA to cells was significantly lowered in the presence of 40 nM PDGF-BB, but not by 40 nM PDGF-AA, showing the specificity of the binding of pPB-HSA to PDGF-β receptors on activated HSC.

3.5. pPB-HSA and renal fibrosis

To examine whether the distribution of pPB-HSA *in vivo* is influenced by an upregulation of PDGF-β receptor expression, we also studied the organ and cellular distribution of pPB-HSA in rats with glomerulosclerosis induced by anti-Thy1.1 IgG. This complement activation-inducing antibody binds selectively to the Thy1.1 antigen present on mesangial cells. At 1–2 days after injection of this antibody to rats, the glomeruli are damaged due to complement activation, resulting in mesangiolysis and leakage of proteins into the urine [28]. We assessed that the total protein concentrations in the urine was maximal at 2 days after injection (378 ± 36 mg/24 hr as compared to 4 ± 3 mg/24 hr in healthy rats). In the subsequent phase, glomerular integrity is restored and proteinuria declines [28]. We measured that at 21 days after injection the urinary protein concentration in these rats was decreased to 62 ± 18 mg/24 hr. At this time point, the glomeruli were repopulated by mesangial cells (anti-Thy1.1 positive cells) and also an enhanced deposition of extracellular matrix was found (collagen type III). These observations correlate with previous studies [28]. In these kidneys, the PDGF-β receptor was expressed in the glomeruli as depicted in Fig. 6A. Ten to 30 min after i.v. administration of the modified albumin pPB-HSA, HSA staining was clearly demonstrated within the glomeruli reflecting the localization of our drug carrier (Fig. 6B). This pPB-HSA was not found in the vascular lumen of the glomeruli, but associated with cells as observed in paraffin embedded sections (not shown). Also, pPB-HSA was not removed by perfusion of the kidneys confirming the cellular binding. Some of the tubular cells also contained the protein, which may indicate leakage of the modified albumin and uptake by the tubular cells of the particular nephrons. After i.v. injection of unmodified HSA to these rats, staining for albumin in the perfused kidney was not detected in glomeruli at 10 or 30 min after administration (Fig. 6C). After double staining of the kidney sections of the rats that received pPB-HSA, co-localization of the HSA staining was found with α-smooth muscle actin positive cells in the glomeruli (Fig. 6D).

3.6. pPB-HSA and normal rats

Although the PDGF-β-receptor was not immunohistochemically detectable in healthy livers, pPB-HSA predominantly distributed to the livers in normal rats (Fig. 7). Gamma camera studies demonstrated that already at 2–3 min after i.v. injection, ^{123}I-pPB-HSA was found in the liver region. The hepatic radioactivity increased in time until 7 min after dosing after which the gamma image was basically unchanged (Fig. 7A). Although the overall distribution pattern was similar to the pattern of pPB-HSA in BDL rats, the radioactive area of maximal intensity was significantly smaller than in BDL3 rats. The latter may be related to the increased weight of the bile duct ligated

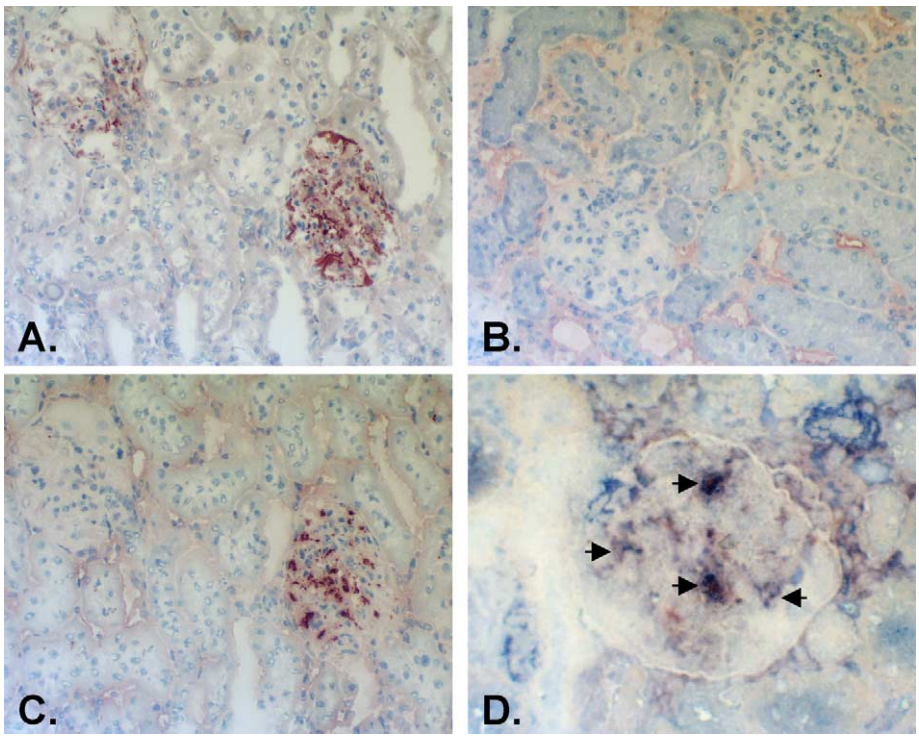

Fig. 6. (A) The renal localization of the PDGF-β receptor in rats with kidney fibrosis at 21 days after i.v. injection of anti-Thy1.1 IgG. (B, C) Immunohistochemical staining for HSA in these fibrotic rats receiving pPB-HSA (B) or HSA (C) at 10 min after i.v. administration. Note that in glomeruli with a higher expression of PDGF-β receptor, more uptake of pPB-HSA was found (A and B are consecutive sections showing the same glomeruli). (D) Co-localization of pPB-HSA (red staining) with α-smooth muscle actin positive cells (blue staining) as indicated by arrows. Original magnifications: 200× (A–C), and 400× (D).

livers. After injection of ^{123}I-HSA, a more general distribution pattern was found in the normal rats with a hot spot in the cardiac region. The radioactive area was greater and its intensity was less as compared to pPB-HSA. Some ^{123}I-HSA was found in the liver region, but this liver-associated radioactivity remained clearly lower than after injection of ^{123}I-pPB-HSA at all time points measured.

Quantitative ^{125}I-tissue measurements at 10 min after i.v. administration in the healthy rats showed that $43 \pm 4\%$ of the dose of pPB-HSA was found in the liver and $37 \pm 3\%$ in the blood (Fig. 7B). Some activity was associated with the kidneys and spleen (in total $5.5 \pm 1.3\%$). In contrast, ^{125}I-HSA was detected only in the blood of these rats. We also studied the distribution of the growth factor PDGF-BB itself in normal rats (Fig. 7B). This PDGF-BB distributed to the same organs as pPB-HSA in normal rats. PDGF-BB was almost completely cleared from the blood at 10 min after administration, and $78 \pm 6\%$ of the ^{125}I-PDGF-BB dose accumulated in the liver, while some PDGF-BB distributed to kidneys, lungs and spleen (together $20 \pm 2\%$ of the dose).

Immunohistochemical localization studies in normal rats revealed that pPB-HSA was detectable in the liver. Within the liver, pPB-HSA was mainly present in hepatocytes at 10 min after i.v. injection (Fig. 7C), because the localization of pPB-HSA correlated with the staining for the hepatocyte plasma membrane marker NTCP. In contrast to diseased kidneys (Fig. 6), no staining was found in the kidneys of normal rats.

4. Discussion

A new approach to treat fibrotic diseases is proposed in the present study, that is, drug targeting to the proliferating fibroblast-like cells. A common feature in fibrotic processes is the upregulation of the PDGF-β receptor on fibroblasts. Therefore, we decided to design a drug carrier directed to this particular receptor. Although PDGF itself may display the most optimal interaction with the receptor, the use of this growth factor as a carrier molecule is not feasible because of its biological effects that in fact would enhance the progression of fibrosis. Therefore, we constructed the peptide C*SRNLIDC* containing the amino acids involved in the binding of the PDGF-B chain to its receptor [17], attached it to albumin, and studied its affinity for the PDGF-β receptor. Since the amino acid sequences responsible for the receptor binding are identical in rats and humans, the newly designed carrier may also be applicable in the human situation. Attempts to prepare albumin with cyclic octapeptides containing the binding site of the PDGF-A chain, i.e. arginine–lysine–lysine–proline [34], failed.

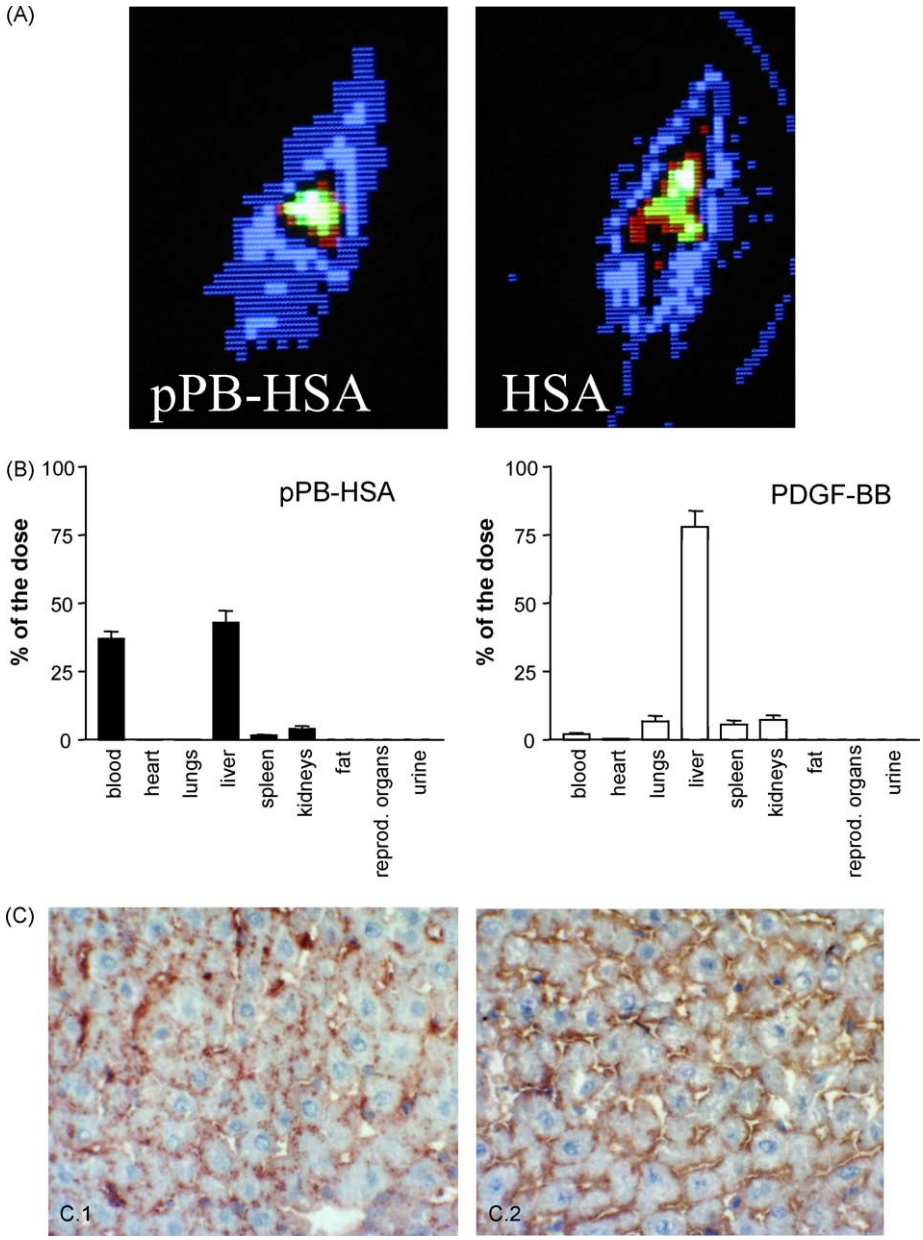

Fig. 7. Distribution of pPB-HSA in normal rats. (A) Representative gamma camera image ($t = 15$–30 min) of the rats after i.v. administration of ^{123}I-pPB-HSA or ^{123}I-HSA. (B) The organ distribution of ^{125}I-pPB-HSA and ^{125}I-PDGF-BB in normal rats at 10 min after i.v. injection. The results are expressed as the mean ± SD. (C) Immunohistochemical detection of pPB-HSA (C.1) in the liver using anti-HSA antibodies. NTCP staining demonstrates the localization of hepatocytes (C.2). Original magnification: 200×. N = 3.

The present study clearly showed that the C*SRNLIDC*-peptide modified albumin, pPB-HSA, binds to PDGF receptors. First, data were obtained in NIH/3T3 fibroblast cultures that showed displacement of ^{125}I-PDGF-BB from its receptor by increasing concentrations of pPB-HSA. Secondly, pPB-HSA bound specifically to activated HSC, a (myo)fibroblast in the liver with an upregulated expression of PDGF-β receptor [11]. We show in the present study that this cellular interaction was inhibited by PDGF-BB as well as by the growth factor receptor blocking agents suramin and trapidil [26,27], but not by PDGF-AA. Third, the PDGF-induced cell proliferation was inhibited to a certain extent by pPB-HSA. We were also able to show the *in vivo* distribution of pPB-HSA to fibroblast-like cells during fibrotic diseases of the liver and kidney. These models are characterized by proliferation of such cells and by high PDGF-receptor expression as confirmed in the present study. Activated HSC are the hepatic cells that display a high PDGF-β receptor expression during liver fibrosis [11], and these cells largely accumulated the pPB-HSA carrier. In the kidney, the mesangial cells upregulate the expression of the PDGF-β receptor during fibrogenesis [13,14], and also these cells accumulated pPB-HSA. Co-localization with the PDGF-β receptor expression and the respective cell markers was clearly demonstrated in our studies.

The cyclic peptides attached to HSA displayed a better interaction with the receptor than the cyclic peptide alone, i.e. the uncoupled peptides were not able to compete with the cellular binding of ^{125}I-PDGF-BB within the concentration range studied (Fig. 1). Apparently, exposition of more than one cyclic peptide on the albumin molecule more closely mimics the binding sites present in the PDGF molecule, which is a dimeric molecule [35]. Covalent attachment of a number of cyclic peptides to a backbone, like HSA, may provide a better presentation to the receptor than the solubilized monomeric peptides. Another advantage of coupling the peptides to a macromolecule like HSA is the prevention of rapid renal excretion that usually occurs with low molecular weight compounds.

The cyclic peptide used by us accommodates the amino acids responsible for the binding of PDGF-BB to its receptor, whereas part of the growth factor responsible for the intracellular signalling is not incorporated. The lack of cell proliferating activity of pPB-HSA on fibroblasts was confirmed in studies measuring [^{3}H]-thymidine incorporation in 3T3 fibroblast cultures. Furthermore, pPB-HSA was able to inhibit proliferation induced by PDGF-BB. Therefore, it is speculated that this pPB-HSA may not only serve as a drug carrier but also may have some intrinsic therapeutic effects because of the antagonistic effects on the PDGF receptor and the pivotal role of PDGF during fibrotic and sclerotic diseases. However, this remains to be established *in vivo*. Anti-fibrotic effects of other PDGF-receptor antagonists have been reported [36].

The *in vivo* distribution pattern of pPB-HSA correlated closely with the organ distribution of PDGF-BB. Like PDGF-BB, pPB-HSA accumulated predominantly in the liver with no apparent difference between normal and diseased rats. However, the intrahepatic distribution of pPB-HSA differed essentially between normal and diseased rats. Whereas in BDL livers pPB-HSA mainly bound to non-parenchymal cells, the distribution was confined to parenchymal cells in normal livers. The rapid uptake of PDGF-BB in normal livers confirms previous kinetic data on PDGF with regard to a predominant distribution to liver and kidneys [37–39]. This distribution reflects the degradation and elimination of PDGF from plasma, rather than being the sites of biological activity. The elimination of pPB-HSA in healthy animals may also not occur via the PDGF α- or β-receptors, but the observed binding to hepatocytes may represent clearance of pPB-HSA possibly following a similar route as the growth factor PDGF, for instance via opsonization by α_2-macroglobulin [39]. During fibrogenesis this elimination route seems to be by-passed by its binding to the specific PDGF-β receptors that are abundantly expressed then on the proliferating fibroblasts.

The interaction of the macromolecule pPB-HSA with the PDGF receptor makes this modified albumin in principle applicable as a drug carrier to any fibrotic organ, in particular to the fibroblast-like cells in these organs. In this study, the homing was tested in rat models of liver and kidney fibrosis, but it may also be applicable in other proliferative and inflammatory diseases associated with an upregulation of the PDGF-β receptor, such as for instance pancreatic fibrosis, atherosclerosis, and inflammatory joint disease. The fibroblasts are the major pathogenic cells, responsible for the production of the excessive amounts and deposition of aberrant forms of extracellular matrix, and therefore these cells are the main target cells for anti-fibrotic drugs. The albumin backbone of the carrier allows the coupling of such drugs in addition to the cyclic peptide homing devices. Drugs can be coupled via spacer moieties that allow release of the drug either at the inside or at the outside of the target cells [40]. The latter is the technology of choice here since the pPB-HSA carrier seemed to remain bound to the plasma membranes of stellate cells (Fig. 5). Many new anti-fibrotic agents emerge but their effects *in vivo* are often disappointing either due to a limited uptake in the target cell or to unwanted side effects [41]. pPB-HSA may be used as a fibroblast-selective carrier for these drugs that allows an effective pharmacotherapeutical intervention in fibrosis of the liver or the kidney.

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References

[1] Powell DW, Mifflin RC, Valentich JD, Crowe SE, Saada JI, West AB. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol 1999;277:C1–C19.
[2] Heldin C-H. Simultaneous induction of stimulatory and inhibitory signals by PDGF. FEBS Lett 1997;410:17–21.
[3] Claesson-Welsh L. Signal transduction by the PDGF receptors. Progr Growth Factor Res 1994;5:37–54.

[4] Pinzani M, Marra F. Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis 2001;21:397–416.
[5] Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, Eriksson U. PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. Nat Cell Biol 2000;2:302–9.
[6] Gilbertson DG, Duff ME, West JW, Kelly JD, Sheppard PO, Hofstrand PD, Gao Z, Shoemaker K, Bukowski TR, Moore M, Feldhaus AL, Humes JM, Palmer TE, Hart CE. Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF alpha and beta receptor. J Biol Chem 2001;276:27406–14.
[7] Bergsten E, Uutela M, Li X, Pietras K, Ostman A, Heldin CH, Alitalo K, Eriksson U. PDGF-D is a specific, protease-activated ligand for the PDGF beta-receptor. Nat Cell Biol 2001;3:512–6.
[8] Larochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, Sullivan C, Boldog FL, Yang M, Vernet C, Burgess CE, Fernandes E, Deegler LL, Rittman B, Shimkets J, Shimkets RA, Rothberg JM, Lichenstein HS. PDGF-D, a new protease-activated growth factor. Nat Cell Biol 2001;3:517–21.
[9] Friedman SL. The virtuosity of hepatic stellate cells. Gastroenterology 1999;117:1244–6.
[10] Win KM, Charlotte F, Mallat A, Cherqui D, Martin N, Mavier P, Preaux AM, Dhumeaux D, Rosenbaum J. Mitogenic effect of transforming growth factor-β1 on human Ito cells in culture—evidence for mediation by endogenous platelet-derived growth factor. Hepatology 1993;18:137–45.
[11] Wong L, Yamasaki G, Johnson RJ, Friedman SL. Induction of β-platelet-derived growth factor receptor in rat hepatic lipocytes during cellular activation *in vivo* and in culture. J Clin Invest 1994;94:1563–9.
[12] Pinzani M, Milani S, Herbst H, DeFranco R, Grappone C, Gentilini A, Caligiuri A, Pellegrini G, Ngo DV, Romanelli RG, Gentilini P. Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. Am J Pathol 1996;148:785–800.
[13] Johnson RJ, Floege J, Yoshimura A, Iida H, Couser WG, Alpers CE. The activated mesangial cell: a glomerular "myofibroblast"? J Am Soc Nephrol 1992;2:S190–7.
[14] Iida H, Seifert R, Alpers CE, Gronwald RGK, Phillips PE, Pritzl P, Gordon K, Gown AM, Ross R, Bowen-Pope DF, Johnson RJ. Platelet-derived growth factor (PDGF) and PDGF receptor are induced in mesangial proliferative nephritis in the rat. Proc Natl Acad Sci USA 1991;88:6560–4.
[15] Matsuda M, Shikata K, Makino H, Sugimoto H, Ota K, Akiyama K, Hirata K, Ota Z. Gene expression of PDGF and PDGF receptor in various forms of glomerulonephritis. Am J Nephrol 1997;17:25–31.
[16] Kliem V, Johnson RJ, Alpers CE, Yoshimura A, Couser WG, Koch KM, Floege J. Mechanisms involved in the pathogenesis of tubulointerstitial fibrosis in 5/6-nephrectomized rats. Kidney Int 1996;49:666–78.
[17] Clements JM, Bawden LJ, Bloxidge RE, Catlin G, Cook AL, Craig S, Drummond AH, Edwards RM, Fallon A, Green DR, Hellewell PG, Kirwin PM, Nayee PD, Richardson SJ, Brown D, Chahwala SB, Snary M, Winslow D. Two PDGF-B chain residues, arginine 27 and isoleucine 30, mediate receptor binding and activation. EMBO J 1991;10:4113–20.
[18] Koivunen E, Wang B, Ruoslahti E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. Biotechnology (NY) 1995;13:265–70.
[19] Marcelino J, McDevitt CA. Attachment of articular cartilage chondrocytes to the tissue form of type VI collagen. Biochim Biophys Acta 1995;1249:180–8.
[20] Kok RJ, Schraa AJ, Bos EJ, Moorlag HE, Asgeirsdottir SA, Everts ME, Meijer DKF, Molema G. Preparation and functional evaluation of RGD-modified proteins as alpha(v)beta(3) integrin directed therapeutics. Bioconjug Chem 2002;13:128–35.
[21] Beljaars L, Molema G, Schuppan D, Geerts A, De Bleser PJ, Weert B, Meijer DKF, Poelstra K. Successful targeting of albumin to rat hepatic stellate cells using albumin modified with cyclic peptides that recognize the collagen type VI receptor. J Biol Chem 2000;275:12743–51.
[22] Mather SJ, Ward BC. High efficiency iodination of monoclonal antibodies for radiotherapy. J Nucl Med 1987;28:1034–6.
[23] Bolton AE, Hunter WM. The labeling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem J 1973;133:529–39.
[24] Engström U, Engström A, Ernlund A, Westermark B, Heldin CH. Identification of a peptide antagonist for platelet-derived growth factor. J Biol Chem 1992;267:16581–7.
[25] Beljaars L, Olinga P, Molema G, De Bleser P, Geerts A, Groothuis GMM, Meijer DKF, Poelstra K. Characteristics of the hepatic stellate cell-selective carrier mannose 6-phosphate modified albumin (M6P(28)-HSA). Liver 2001;21:320–8.
[26] Heidaran MA, Mahadevan D, Larochelle WJ. Beta PDGFR-IgG chimera demonstrates that human β PDGFR Ig-like domains 1 to 3 are sufficient for high affinity PDGF BB binding. FASEB J 1995;9:140–5.
[27] Gesualdo L, DiPaolo S, Ranieri E, Schena FP. Trapidil inhibits human mesangial cell proliferation: effect on PDGF β-receptor binding and expression. Kidney Int 1994;46:1002–9.
[28] Bagchus WM, Hoedemaeker PJ, Rozing J, Bakker WW. Glomerulonephritis induced by monoclonal anti-Thy1.1 antibodies. A sequential histological and ultrastructural study in the rat. Lab Invest 1986;55:680–7.
[29] Poelstra K, Hardonk MJ, Koudstaal J, Bakker WW. Intraglomerular platelet aggregation and experimental glomerulonephritis. Kidney Int 1990;37:1500–8.
[30] Haas M, De Zeeuw D, Van Zanten A, Meijer DKF. Quantification of renal low-molecular-weight protein handling in the intact rat. Kidney Int 1993;43:949–54.
[31] Wright TL, Roll FJ, Jones AL, Weisiger RA. Uptake and metabolism of polymerized albumin by rat liver. Gastroenterology 1988;94:443–52.
[32] Toth CA, Thomas P. Liver endocytosis and Kupffer cells. Hepatology 1992;16:255–66.
[33] Yoshioka T, Yamamoto K, Kobashi H, Tomita M, Tsuji T. Receptor-mediated endocytosis of chemically modified albumins by sinusoidal endothelial cells and Kupffer Cells in rat and human liver. Liver 1994;14:129–37.
[34] Fenstermaker RA, Poptic E, Bonfield TL, Knauss TC, Corsillo L, Piskurich JE, Gelfand C, DiCorleto PE, Kaetzel DM. A cationic region of the platelet-derived growth factor (PDGF) A-chain (Arg^{159}-Lys^{160}-Lys^{161}) is required for receptor binding and mitogenic activity of the PDGF-AA homodimer. J Biol Chem 1993;268:10482–9.
[35] Fretto LJ, Snape AJ, Tominson JE, Seroogy JJ, Wolf DL, Larochelle WJ, Giese NA. Mechanism of platelet-derived growth factor (PDGF) AA, AB, and BB binding to alpha and beta receptor. J Biol Chem 1993;268:3625–31.
[36] Floege J, Ostendorf T. Platelet-derived growth factor: a new clinical target on the horizon. Kidney Int 2001;59:1592–3.
[37] Cohen AM, Soderberg C, Thomason A. Plasma clearance and tissue distribution of recombinant human platelet-derived growth factor (B-chain homodimer) in rats. J Surg Res 1990;49:447–52.
[38] Bowen-Pope DF, Malpass TW, Foster DM, Ross R. Platelet-derived growth factor *in vivo*: levels, activity, and rate of clearance. Blood 1984;64:458–69.
[39] Lamarre J, Wollenberg GK, Gonias SL, Hayes MA. Cytokine binding and clearance properties of proteinase-activated alpha 2-macroglobulins. Lab Invest 2000;65:3–14.
[40] Soyez H, Schacht E, Van Der Kerken S. The crucial role of spacer groups in macromolecular prodrug design. Adv Drug Deliv Rev 1996;21:81–106.
[41] Beljaars L, Meijer DKF, Poelstra K. Targeting hepatic stellate cells for cell-specific treatment of liver fibrosis. Front Biosci 2002;7:E214–22.