

PDGF-Receptor β -Targeted Adenovirus Redirects Gene Transfer from Hepatocytes to Activated Stellate Cells

Marieke H. Schoemaker,^{†,‡} Marianne G. Rots,[‡] Leonie Beljaars,[§]
Arjen Y. Ypma,[‡] Peter L. M. Jansen,[†] Klaas Poelstra,[§] Han Moshage,[†] and
Hidde J. Haisma^{*,‡}

Department of Gastroenterology and Hepatology, Department of Therapeutic Gene Modulation, and Department of Pharmacokinetics and Drug Delivery, University of Groningen and University Medical Center Groningen, Groningen, The Netherlands

Received September 3, 2007; Revised Manuscript Received December 5, 2007; Accepted December 10, 2007

Abstract: Chronic liver damage may lead to liver fibrosis. In this process, hepatic activated stellate cells are the key players. Thus, activated stellate cells are attractive targets for antifibrotic gene therapy. Recombinant adenovirus is a promising vehicle for delivering therapeutic genes to liver cells. However, this vector has considerable tropism for hepatocytes and Kupffer cells. The aim of this study is therefore to retarget the adenovirus to the activated stellate cells while reducing its affinity for hepatocytes. We constructed a fusion protein with affinity for both the adenovirus and the platelet derived growth factor-receptor β (PDGF-R β). In contrast to other cells, the PDGF-R β is highly expressed on activated stellate cells. The targeting moiety, the PDGF peptide CSRNLIDC, was cloned in front of the single-chain antibody fragment (S11) directed against the adenoviral knob. This fusion protein enhanced adenoviral gene transfer in both 3T3 fibroblasts and primary isolated activated rat stellate cells by 10–60-fold. A fusion protein with a scrambled PDGF peptide (CIDNLSRC) did not accomplish this effect. Importantly, the PDGF-R β -retargeted adenovirus showed a 25-fold reduced tropism for primary rat hepatocytes. Our novel approach demonstrates that therapeutic genes can be selectively directed to stellate cells. This opens new possibilities for the treatment of liver fibrosis.

Keywords: scFv; liver fibrosis; gene targeting; PDGF-receptor; adenovirus

Introduction

Chronic liver injury may induce liver fibrosis leading to organ dysfunction.¹ Liver fibrosis is characterized by excessive deposition of scar tissue.² The central effector cell during

liver fibrosis is the hepatic stellate cell, which is the major producer of extracellular matrix components. During liver injury, stellate cells are exposed to paracrine stimuli from injured hepatocytes, endothelial cells, and Kupffer cells.³ This process initiates the activation and proliferation of stellate cells, which is considered as a key event in fibrosis. After the onset of liver fibrosis, the expression of the platelet derived growth factor-receptor β (PDGF-R β) is strongly enhanced on activated stellate cells. The production of its ligand PDGF is increased as well.⁴ Current pharmacological approaches for the treatment of liver fibrosis are not effective. The only adequate therapy is a liver transplantation. Antifibrotic therapies that specifically act on activated stellate

* To whom correspondence should be addressed. Mailing address: Department of Therapeutic Gene Modulation, University of Groningen, Ant. Deusinglaan 1, 9713 AV Groningen, The Netherlands. Tel: 31-50-3637866. Fax: 31-50- 3633247. E-mail: h.j.haisma@rug.nl.

[†] Department of Gastroenterology and Hepatology.

[‡] Department of Therapeutic Gene Modulation.

[§] Department of Pharmacokinetics and Drug Delivery.

- (1) Friedman, S. L. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J. Biol. Chem.* **2000**, 275 (4), 2247–2250.
- (2) Rojkind, M.; Giambrone, M. A.; Biempica, L. Collagen types in normal and cirrhotic liver. *Gastroenterology* **1979**, 76 (4), 710–719.

(3) Kawada, N. The hepatic perisinusoidal stellate cell. *Histol. Histopathol.* **1997**, 12 (4), 1069–1080.

(4) Friedman, S. L. Liver fibrosis—from bench to bedside. *J. Hepatol.* **2003**, 38 (Suppl. 1), S38–S53.

cells may enhance effectiveness. Targeting of therapeutic genes may represent such a novel therapy. Previous studies have shown that apoptosis of activated stellate cells may contribute to the resolution of fibrosis.^{5,6} Induction of apoptosis seems therefore a promising therapeutic strategy. Apoptosis can be accomplished by inhibiting the activation of the transcription factor NF- κ B using adenoviruses expressing an I κ B dominant negative protein.⁷ However, a therapeutic effect can only be achieved when apoptosis is induced exclusively in activated stellate cells. Induction of apoptosis in hepatocytes would most likely be deleterious in fibrotic livers. Therefore, the present study describes a novel tool to deliver therapeutic genes specifically to activated stellate cells. The induction of PDGF-R β on cell membranes of activated stellate cells prompted us to examine PDGF as a targeting moiety. PDGF is a dimeric molecule composed of A and/or B chains and induces proliferation of stellate cells. The PDGF-R β exclusively binds the B-chain of PDGF.⁸ Activated stellate cells predominantly express PDGF-R β on their cell membranes, whereas liver endothelial cells only contain PDGF-R α .⁹ Thus, activated stellate cells specifically bind PDGF-BB.^{8,9} In addition, the concentration of the PDGF-R β on activated stellate cells is much higher during liver fibrosis compared to other PDGF-R β -positive cells. Homing of a PDGF-peptide to the PDGF-R β has previously been demonstrated using PDGF peptide-modified albumin as a drug carrier.¹⁰ The arginine (R) and isoleucine (I) of this novel peptide (CSRNLIDC) are responsible for binding to the PDGF-R β .¹¹ Selective binding of the peptide to the PDGF-R β on activated stellate cells has been demonstrated *in vivo*. In addition, it has been shown that

this PDGF-peptide does not initiate a signaling cascade upon binding to PDGF-R.¹⁰ In general, adenovirus attaches to cells by binding of the knob of the fiber coat protein to the coxsackievirus and adenovirus receptor (CAR).¹² Cell entry is subsequently mediated by binding of the penton base coat protein to integrins on the cell membrane.¹³ In the present study, retargeting of recombinant adenoviruses to the PDGF-R β on activated stellate cells is investigated. We created a fusion protein, which consists of a PDGF-R β -recognizing peptide.¹⁰ coupled to a single-chain antibody fragment with affinity for the knob of recombinant adenovirus.¹⁴ Previously, this ligand-directed strategy was used successfully by selective targeting to the epidermal growth factor receptor.^{14,15} Comparative evaluation of gene delivery devices in hepatic stellate cells revealed that these cells are more susceptible to adenoviral-mediated gene transfer as compared to nonviral vectors.¹⁶ In contrast to a normal liver, after the onset of liver fibrosis adenoviral transduction efficiency is much higher in nonparenchymal cells than in hepatocytes.¹⁷ Nonetheless, hepatocytes are still infected.¹⁸ For certain antifibrotic therapies, selective gene delivery in activated stellate cells is crucial, thereby reducing gene expression in hepatocytes. We present here the first tool to achieve that goal.

Materials and Methods

Cell Culture. NIH/3T3 fibroblasts were cultured in DMEM (Gibco, Paisley, Scotland), 5% fetal bovine serum (BIO Whittaker Europe, Verviers, Belgium), glutamine, penicillin, and streptomycin (Gibco). HCS-T6 cells (immortalized rat liver stellate cells) were kindly provided by

- (5) Iredale, J. P.; Benyon, R. C.; Pickering, J.; McCullen, M.; Northrop, M.; Pawley, S.; Hovell, C.; Arthur, M. J. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J. Clin. Invest.* **1998**, *102* (3), 538–549.
- (6) Wright, M. C.; Issa, R.; Smart, D. E.; Trim, N.; Murray, G. I.; Primrose, J. N.; Arthur, M. J.; Iredale, J. P.; Mann, D. A. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. *Gastroenterology* **2001**, *121* (3), 685–698.
- (7) Lang, A.; Schoonhoven, R.; Tuvia, S.; Brenner, D. A.; Rippe, R. A. Nuclear factor kappaB in proliferation, activation, and apoptosis in rat hepatic stellate cells. *J. Hepatol.* **2000**, *33* (1), 49–58.
- (8) Pinzani, M.; Marra, F. Cytokine receptors and signaling in hepatic stellate cells. *Semin. Liver Dis.* **2001**, *21* (3), 397–416.
- (9) Heldin, P.; Pertoft, H.; Nordlinder, H.; Heldin, C. H.; Laurent, T. C. Differential expression of platelet-derived growth factor alpha- and beta- receptors on fat-storing cells and endothelial cells of rat liver. *Exp. Cell Res.* **1991**, *193* (2), 364–369.
- (10) Beljaars, L.; Weert, B.; Geerts, A.; Meijer, D. K.; Poelstra, K. The preferential homing of a platelet derived growth factor receptor-recognizing macromolecule to fibroblast-like cells in fibrotic tissue. *Biochem. Pharmacol.* **2003**, *66* (7), 1307–1317.
- (11) Clements, J. M.; Bawden, L. J.; Bloxidge, R. E.; Catlin, G.; Cook, A. L.; Craig, S.; Drummond, A. H.; Edwards, R. M.; Fallon, A.; Green, D. R. Two PDGF-B chain residues, arginine 27 and isoleucine 30, mediate receptor binding and activation. *EMBO J.* **1991**, *10* (13), 4113–4120.
- (12) Bergelson, J. M.; Cunningham, J. A.; Droguett, G.; Kurt-Jones, E. A.; Krithivas, A.; Hong, J. S.; Horwitz, M. S.; Crowell, R. L.; Finberg, R. W. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* **1997**, *275* (5304), 1320–1323.
- (13) Einfeld, D. A.; Schroeder, R.; Roelvink, P. W.; Lizonova, A.; King, C. R.; Kovesdi, I.; Wickham, T. J. Reducing the native tropism of adenovirus vectors requires removal of both CAR and integrin interactions. *J. Virol.* **2001**, *75* (23), 11284–11291.
- (14) Haisma, H. J.; Grill, J.; Curiel, D. T.; Hoogeland, S.; van, B. V.; Pinedo, H. M.; Gerritsen, W. R. Targeting of adenoviral vectors through a bispecific single-chain antibody. *Cancer Gene Ther.* **2000**, *7* (6), 901–904.
- (15) Watkins, S. J.; Mesyanzhinov, V. V.; Kurochkina, L. P.; Hawkins, R. E. The 'adenobody' approach to viral targeting: specific and enhanced adenoviral gene delivery. *Gene Ther.* **1997**, *4* (10), 1004–1012.
- (16) Weiskirchen, R.; Kneifel, J.; Weiskirchen, S.; van de, L. E.; Kunz, D.; Gressner, A. M. Comparative evaluation of gene delivery devices in primary cultures of rat hepatic stellate cells and rat myofibroblasts. *BMC Cell Biol.* **2000**, *1*, 4.
- (17) Yu, Q.; Que, L. G.; Rockey, D. C. Adenovirus-mediated gene transfer to nonparenchymal cells in normal and injured liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2002**, *282* (3), G565–G572.
- (18) Nakamura, T.; Akiyoshi, H.; Saito, I.; Sato, K. Adenovirus-mediated gene expression in the septal cells of cirrhotic rat livers. *J. Hepatol.* **1999**, *30* (1), 101–106.

Dr. S. L. Friedman (Mount Sinai School of Medicine, New York, NY) and maintained as described previously.¹⁹ Primary rat hepatocytes and stellate cells were isolated and cultured as described previously.^{20,21} Rat hepatic stellate cells (HSCs) were cultured for at least 10 days. At this stage, these cells have an activated phenotype. All cells were cultured in a humidified atmosphere at 37 °C/5% CO₂.

Adenovirus AdTL. Cesium chloride-purified E1- and E3-deleted adenovirus type 5 was obtained by standard techniques.²² This recombinant adenovirus (AdTL) contains expression cassettes for green fluorescent protein (GFP) and firefly luciferase under the control of the CMV promoter.²³

Construction of CSRNLC-S11 and CIDNLSRC-S11 Fusion Proteins. For the construction of fusion proteins, pUC119-pelB-S11-myhis vector was used (a generous gift of Dr. R. E. Hawkins, Bristol University, U.K.). Downstream of the coding sequence for the single-chain antibody fragment (scFv) against adenoviral fiber knob (S11), a *Myc* tag and a histidine-tag (His6) sequence were present. The PelB leader was located upstream of the coding sequence for the fusion protein and allowed the isolation of the protein out of the periplasmic space. A sequence encoding the PDGF-BB peptide (amino acids CSRNLC)¹⁰ was introduced between the PelB leader and the S11 sequence using oligonucleotides. The sense oligo 5'-CATGCCCTGCTCGCGGAACCT-CATCGATTGTGGCGGCGGCAGCTC-3' was annealed to antisense oligo 5'-CATGGAGCTGCCGCCACAATC-GATGAGGTTCC GCGAGCAGGG-3'. This resulted in a DNA fragment with overlap extensions similar to the restriction sequence of *NcoI*. The bold nucleotides represent the DNA sequences encoding the PDGF-BB peptide CSRNLC. The glycine linker is presented as an underlined sequence and separates the CSRNLC-peptide from S11. The DNA fragment was phosphorylated and cloned into dephosphorylated, *NcoI*-restricted pUC119-pelB-S11-myhis vector. DNA encoding for a scrambled peptide (amino acids CIDNLSRC) was introduced by PCR upstream of the S11 sequence. Using pUC119-pelB-S11-myhis as a template, PCR was performed with Taq DNA polymerase (Qiagen Inc.,

Valencia, CA) and the following primers, forward 5'-TTAACCATGGCCTGCATAGACAACCTCTC-GAGATGT GGCGGCGGCAGCTCTATGGCCCAGGT-GCAACTGCAGC-3', and reverse 5'-TTAATCT AGATTAT-TAATGGTGATGATGG-3'. *E. coli* JM109 were transformed with pUC119pelBS11-myhis, which included the sequence CSRNLC or CIDNLSRC.

Expression and Purification of the Fusion Proteins and Adenovirus Serotype 5 Knob Domain. JM 109 *E. coli*, transfected with plasmids encoding for CSRNLC-S11 or CIDNLSRC-S11, were grown overnight at 37 °C (with shaking at 200 rpm) in 2TY medium supplemented with 100 µg/mL of ampicillin and 1% glucose. The overnight culture was diluted 1:100 in 1 L of 2TY medium containing 100 µg/mL of ampicillin and 0.1% glucose. Bacterial suspensions were grown at 37 °C (200 rpm) to an optical density of 0.8–1.0 at 600 nm. Protein expression was induced by addition of 1 mM isopropyl B-D-thiogalactoside (IPTG; Invitrogen, Breda, The Netherlands) followed by an incubation for 4 h at 30 °C (200 rpm). Bacteria were harvested by centrifugation at 6000g (4 °C) for 10 min and resuspended in 20 mL of ice-cold TES buffer (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0). This suspension was incubated for 15 min on ice and subsequently centrifuged at 38000g (4 °C) for 30 min. The pellet was resuspended in 2–5 volumes of ice-cold sonication buffer (50 mM NaH₂PO₄, 300 mM NaCl, pH 7.8) followed by addition of 1 mg/mL of lysozyme (ICN Biomedicals, Aurora, OH) and an incubation step on ice for 30 min. This suspension was sonicated three times for 10 s and centrifuged for 20 min at 10000g (4 °C) after which the supernatant was collected. Purification of His-tagged proteins was performed by nickel–nitrilotriacetic acid (Ni–NTA) affinity chromatography. For this purpose, the supernatant was mixed overnight with 8 mL of Probond Resin Ni–NTA beads (Invitrogen) at 4 °C. This mixture was washed with sonication buffer until O.D. 280 nm was 0.02, after which another wash step was performed with 20 mM imidazole (diluted in sonication buffer). Protein was eluted with 250 mM imidazole. Fractions containing eluted protein were pooled and dialyzed against phosphate buffer saline (PBS) at 4 °C overnight. Expression of the knob domain of adenovirus serotype 5 was induced in *E. coli* M15(pREP4)(PQE30-Ad5knob) (kindly provided by Dr. J. Douglas, University of Alabama, Birmingham, AL) using LB medium with 100 µg/mL of ampicillin and 25 µg/mL of kanamycin and a final concentration of 2 mM IPTG. Cells were harvested by centrifugation at 4000g for 10 min followed by resuspension in sonication buffer and treated as described above.

Western Blot Analysis. SDS–PAGE was performed with 5 µg of protein (under denaturing conditions) on a 10% polyacrylamide gel followed by Western blot analysis with antimyc monoclonal antibody at a dilution of 1:10. This antibody was collected from culture medium of hybridoma cells 9E10 (ATCC, Manassas, VA) after centrifugation at 200g. Rabbit-antimouse horseradish peroxidase (DAKO A/S, Denmark) was used at a dilution of 1:3000. Protein was

- (19) Vogel, S.; Piantedosi, R.; Frank, J.; Lalazar, A.; Rockey, D. C.; Friedman, S. L.; Blaner, W. S. An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. *J. Lipid Res.* **2000**, *41* (6), 882–893.
- (20) Moshage, H.; Casini, A.; Lieber, C. S. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. *Hepatology* **1990**, *12* (3 Pt 1), 511–518.
- (21) Schoemaker, M. H.; Gommans, W. M.; Conde de la, R. L.; Homan, M.; Klok, P.; Trautwein, C.; van, G. H.; Poelstra, K.; Haisma, H. J.; Jansen, P. L.; Moshage, H. Resistance of rat hepatocytes against bile acid-induced apoptosis in cholestatic liver injury is due to nuclear factor-kappa B activation. *J. Hepatol.* **2003**, *39* (2), 153–161.
- (22) He, T. C.; Zhou, S.; da Costa, L. T.; Yu, J.; Kinzler, K. W.; Vogelstein, B. A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95* (5), 2509–2514.
- (23) Alemany, R.; Curiel, D. T. CAR-binding ablation does not change biodistribution and toxicity of adenoviral vectors. *Gene Ther.* **2001**, *8* (17), 1347–1353.

visualized with an AEC-kit according to manufacturer's instructions (Sigma, Steinheim, Germany). Kaleidoscope prestained standards were used (Biorad Laboratories, Hercules, CA) to determine molecular weight.

Analysis of S11 Binding to the Adenoviral Fiber Knob. The S11 binding activity of CSRNLIDC-S11 and CIDNLSRC-S11 was tested by enzyme-linked immunosorbent assay (ELISA). A fusion protein directed against the epidermal growth factor receptor (425-S11) and S11 alone was used as positive control.¹⁴ Proteins were added to a 96-well plate precoated with 1 μ g per well of adenoviral type-5 knob. Immunostaining on adenoviral knob precoated wells served as negative control. ELISA was performed with antimyc monoclonal antibody (diluted 1:10; 1 h) followed by a secondary antibody (rabbit antimouse HRP conjugate (1:3000; 1 h)). 1,2-*o*-Phenylenediamine dihydrochloride (OPD) served as a substrate (DAKO A/S, Denmark). After a wash step, binding of the fusion proteins was measured at 490 nm.

PDGF-Receptor Binding Analysis of CSRNLIDC-S11. Binding of CSRNLIDC-S11 to the PDGF-R-positive HSC-T6 cell line was evaluated with fluorescence-activated cell sorting analysis (FACS). Cells were incubated with medium (control) or 10 μ g of CSRNLIDC-S11 for 1 h at 4 °C. Successively, antimyc antibody (1:10) and PE (phycoerythrin) conjugated goat-antimouse (GAMPE, 1:100; DAKO) were used. Incubation steps were followed by centrifugation for 3 min at 1500 rpm in serum-free medium.

Targeting and Untargeting Assays. NIH/3T3 fibroblasts were used as a model system since they express high levels of PDGF-R β and very low levels of CAR.^{24,25} These cells were plated in 96-well plates (NUNC, Denmark) at a density of 10,000 cells per well and incubated overnight at 37 °C. Primary isolated culture-activated rat stellate cells (HSCs) were plated in 12-well plates until monolayers were subconfluent. Rat hepatocytes were used to investigate untargeting of PDGF-R-retargeted virus. After isolation, hepatocytes were plated on 12-well plates and grown for 5 h as described previously.²⁶ Virus was preincubated with CSRNLIDC-S11 or CIDNLSRC-S11 at a ratio of 1.0×10^9 viral particles per 1 or 10 μ g of protein for 30 min at 37 °C. NIH/3T3 fibroblasts and HSCs were transfected in triplicate wells (in medium containing 2% FBS) with 500 plaque forming units (pfu) or 50 pfu per cell of preincubated AdTL,

respectively. Hepatocytes were transfected in serum-free medium using 50 pfu per cell of preincubated AdTL.

To study the involvement of CAR, cells received 20 μ g/mL of adenoviral type-5 knob for 1 h at 37 °C before addition of (preincubated) virus. Cells were incubated for 24 h in a humidified atmosphere at 37 °C/5% CO₂, after which they were evaluated for GFP expression using a Zeiss Axiovert 25 fluorescence microscope. After another 24 h, cells were harvested in 1 \times cell lysis buffer (Promega, Madison, WI). Gene transfer was determined by measuring luciferase enzyme activity according to manufacturer's instructions (Promega) using the Lumicount (Packard Bioscience Benelux NV, Groningen, The Netherlands) and expressed as luciferase activity per well. Samples were counted for cell number to evaluate toxicity.

For stability studies, virus was preincubated with fusion protein, mixed with mouse serum (final concentration 50%), and incubated at 37 °C for up to 2 h before transfection. Gene transfer was determined as described above.

Statistical Analysis. Results are presented as the mean of at least three independent experiments \pm standard deviation. A Mann-Whitney test was used to determine the significance of differences between two experimental groups. A *P* value of less than 0.001 (*P* < 0.001) was considered to be statistically significant.

Results

Functionality of Fusion Protein CSRNLIDC-S11. To develop a selective gene delivery tool for activated stellate cells, a fusion protein was constructed. This fusion protein consisted of a single-chain antibody fragment (S11) with affinity for the fiber knob of adenovirus type 5 (Figure 1). In front of the coding sequence of S11, oligonucleotides were inserted encoding a peptide (CSRNLIDC) which binds the PDGF-R β . A glycine linker separated both parts of the fusion protein. A scrambled DNA sequence encoding CIDNLSRC was inserted in front of the DNA sequence encoding S11 to produce control fusion protein. This scrambled fusion protein should not bind to the PDGF-R β . After induction in *E. coli* JM109, both proteins were purified using the 6His tag present in the construct. Correct protein expression was detected at 30 kDa for CSRNLIDC-S11 and CIDNLSRC-S11 (Figure 2a) by a monoclonal antibody against the *myc* tag. Fusion proteins were tested for binding to adenoviral fiber knob using ELISA. As shown in Figure 2b, both proteins bound to the adenoviral fiber knob similar to S11, demonstrating functionality of the S11-single-chain antibody fragment even in the context of a fusion protein. Next, binding of CSRNLIDC-S11 to a PDGF-R β positive stellate cell line was subsequently defined by FACS analysis. Addition of CSRNLIDC-S11 to the stellate cell line HSC-T6 resulted in a clear shift of the PE signal compared to the control situation (only antibodies) and S11 alone (data not shown) (Figure 2c). These results confirm previous data that demonstrated binding of the CSRNLIDC peptide to the PDGF-R β .¹⁰

(24) Somasundaram, R.; Schuppan, D. Type I, II, III, IV, V, and VI collagens serve as extracellular ligands for the isoforms of platelet-derived growth factor (AA, BB, and AB). *J. Biol. Chem.* **1996**, *271* (43), 26884–26891.

(25) Hidaka, C.; Milano, E.; Leopold, P. L.; Bergelson, J. M.; Hackett, N. R.; Finberg, R. W.; Wickham, T. J.; Kovsesi, I.; Roelvink, P.; Crystal, R. G. CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. *J. Clin. Invest.* **1999**, *103* (4), 579–587.

(26) Schoemaker, M. H.; Ros, J. E.; Homan, M.; Trautwein, C.; Liston, P.; Poelstra, K.; van, G. H.; Jansen, P. L.; Moshage, H. Cytokine regulation of pro- and anti-apoptotic genes in rat hepatocytes: NF-kappaB-regulated inhibitor of apoptosis protein 2 (cIAP2) prevents apoptosis. *J. Hepatol.* **2002**, *36* (6), 742–750.

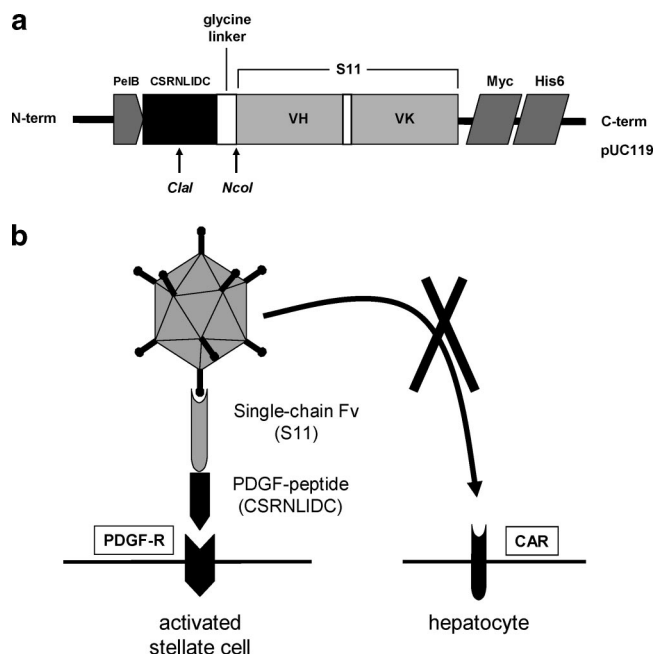


Figure 1. Construction of a fusion protein recognizing both adenoviral type 5 fiber knob and the PDGF-receptor β . The PDGF-receptor β is highly expressed on activated stellate cells. (a) In front of the coding sequence of the single-chain antibody fragment (scFv) S11, the DNA sequence of the PDGF-BB-derived peptide CSRNLIDC was inserted. In between, a glycine linker was located. The PelB leader allows the fusion protein to enter bacterial periplasmic space, which is followed by cleavage of the leader signal. In the periplasmic space, a disulfide bridge can be formed. At the C-terminus of the fusion protein, a histidine- and Myc-tag are situated allowing purification and detection of the fusion protein, respectively. (b) Schematic representation of the gene targeting approach to activated stellate cells. The coating of adenovirus with the fusion protein CSRNLIDC-S11 will block binding to its native receptor CAR. Preincubation of adenovirus with CSRNLIDC-S11 should retarget the virus to the PDGF-receptor β but will not initiate a signaling cascade through this receptor.

CSRNLIDC-S11-Directed Gene Transfer in Fibroblasts. Functionality of our targeting approach was further investigated using NIH/3T3 fibroblasts as a model system. These cells express very high levels of PDGF-R β and low levels of CAR on their cell membranes. Adenoviral gene transfer was measured in terms of luciferase expression. These fibroblasts did not exhibit adenoviral-mediated gene transfer after exposure to unmodified recombinant adenovirus (AdTL) (Figure 3). In addition, the scrambled fusion protein CIDNLSRC-S11 did not result in enhanced adenoviral gene transfer. However, modification of AdTL with CSRNLIDC-S11 dramatically increased luciferase gene expression in a dose-dependent manner (Figure 3). A ratio of 10 μ g of CSRNLIDC-S11 per 10⁹ viral particles resulted in the maximal enhancement of transfection (almost 60-fold). In the presence of higher amounts of protein, the infection rate started to decline (data not shown) as a result of PDGF-R β

competition between unbound protein and re-targeted AdTL.¹⁴ Besides luciferase gene expression, expression of adenovirus-encoded GFP was monitored in these cell cultures. GFP expression correlated with luciferase gene expression (data not shown).

The stability of the binding of the S11-fusion protein to the adenovirus was investigated. Mouse serum (50%) did not affect binding of the conjugated adenovirus. In addition, prolonged incubation of conjugated virus in mouse serum up to 120 min did not diminish gene transfer, indicating stable binding of the S11 to the adenovirus.

CSRNLIDC-S11-Directed Gene Transfer in Activated Stellate Cells. The results of our model system allowed us to examine the transfection efficiency of PDGF-R β -retargeted adenovirus in primary isolated culture-activated stellate cells. Besides high levels of PDGF-R β on their cell membrane,²⁷ activated stellate cells do express CAR.¹⁷ As shown in Figure 4, activated stellate cells were very efficiently transfected with unmodified recombinant adenovirus. This gene transfer was inhibited with the CAR blocking recombinant adenoviral knob. However, preincubation of CSRNLIDC-S11 with AdTL resulted in an enhanced adenoviral-mediated gene transfer in activated stellate cells compared to AdTL alone. This suggests that even in activated stellate cells that are CAR-positive, the transfection of the adenovirus can be enhanced through targeting via the PDGF-R β . Moreover, CAR blockade did not significantly diminish CSRNLIDC-S11-mediated adenoviral gene transfer (Figure 4), indicating that PDGF-R β is responsible for gene transfer rather than CAR.

Untargeting of Primary Hepatocytes. To accomplish specific gene expression in activated stellate cells, a reduced gene expression in hepatocytes is needed since adenovirus has a high tropism for hepatocytes. Therefore, primary cultures of isolated hepatocytes were used to examine our targeting strategy. Hepatocytes express high levels of CAR on their cell membranes, but no PDGF-R β . As expected, nonmodified virus efficiently transfected hepatocytes (Figure 5). This was inhibited through CAR blockade (data not shown). Preincubation of AdTL with the PDGF-R β -specific fusion protein CSRNLIDC-S11 blocked the adenoviral gene transfer in primary hepatocytes almost to background (Figure 5). These data demonstrate a successful blockade of the interaction between adenovirus and hepatocytes by CSRNLIDC-S11.

Discussion

We developed a new tool to specifically deliver genes to activated stellate cells. Stellate cells play a central role in liver fibrosis. Since current pharmacotherapeutic strategies to treat liver fibrosis are not satisfactory, a search for new

(27) Pinzani, M.; Gentilini, A.; Caligiuri, A.; De, F. R.; Pellegrini, G.; Milani, S.; Marra, F.; Gentilini, P. Transforming growth factor-beta 1 regulates platelet-derived growth factor receptor beta subunit in human liver fat-storing cells. *Hepatology* **1995**, *21* (1), 232–239.

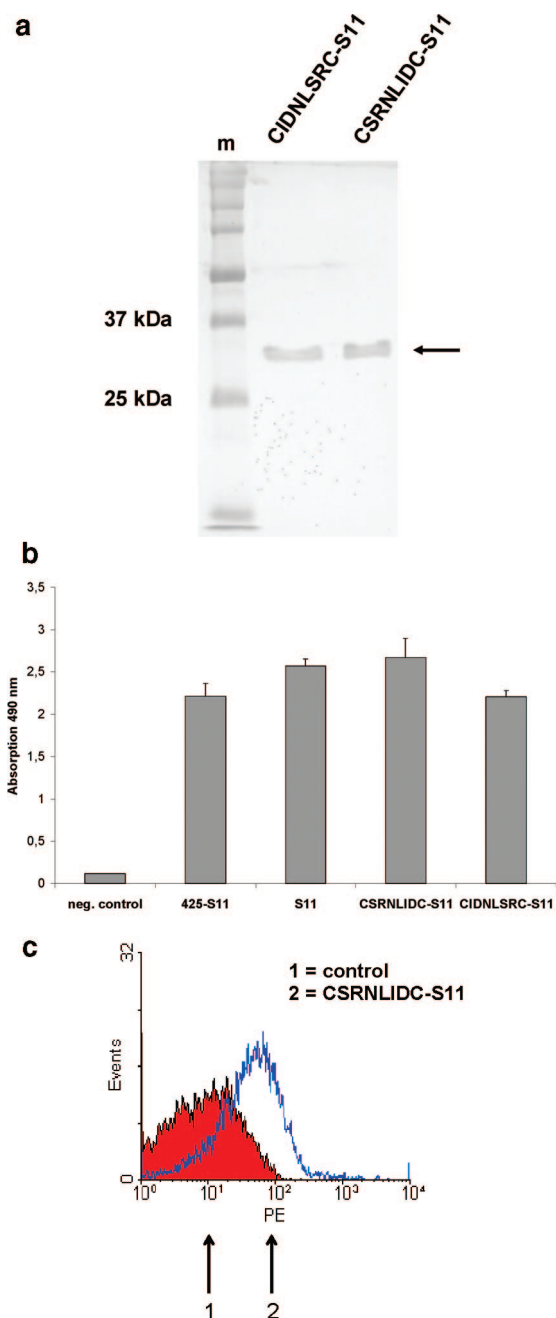


Figure 2. Characterization of the fusion proteins CSRNLIDC-S11 and CIDNLSRC-S11. (a) Western blot analyses of Ni-NTA-purified proteins produced in JM109 *E. coli*. Five micrograms of protein was loaded on a 10% gel, which was detected with anti-Myc antibody (MW of approximately 30 kDa). (b) Binding activity of the S11-single-chain antibody fragment against adenoviral fiber knob using ELISA. S11 alone and a bispecific single-chain antibody directed against the epidermal growth factor receptor (425-S11) served as positive control. Wells that were solely precoated with adenoviral knob served as negative control. (c) The binding of CSRNLIDC-S11 to a PDGF-receptor β positive hepatic stellate cell line (HSC-T6) was evaluated by fluorescence-activated cell sorting. Anti-Myc and PE (phycoerythrin)-conjugated goat-antimouse antibodies were used for detection.

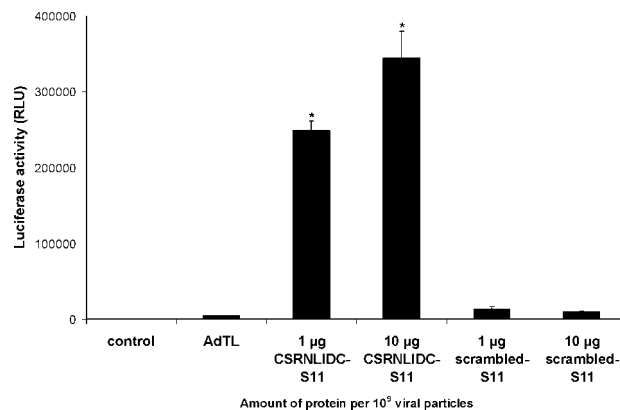


Figure 3. Specific transfection of fibroblasts by PDGF-receptor β -targeted adenovirus. NIH/3T3 fibroblasts express high levels of PDGF-receptor β and low levels of CAR. These cells were used as a model system. 10⁹ viral particles of AdTL were preincubated with or without 1 or 10 μ g of CSRNLIDC-S11 or CIDNLSRC-S11 (scrambled-S11) for 30 min at 37 °C. NIH/3T3 fibroblasts were infected with 500 pfu/cell. Gene transfer is presented as luciferase activity (relative light units) which was measured 48 h after infection. Representative data of five independent experiments are shown with $n = 3$ per condition. * $P < 0.05$ for AdTL + CSRNLIDC-S11 vs AdTL.

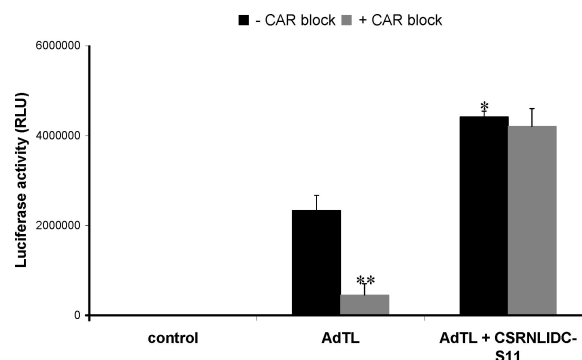


Figure 4. CSRNLIDC-S11 increases adenoviral gene transfer in culture-activated rat stellate cells. Culture-activated stellate cells were infected with PDGF-receptor β retargeted adenovirus. A ratio of 10 μ g of CSRNLIDC-S11 per 10⁹ viral particles (AdTL) was used. Activated stellate cells were infected with 50 pfu/cell in the presence or absence of 20 μ g/mL fiber knob. Soluble fiber knob was used to block CAR. Gene transfer is presented as luciferase activity (relative light units) which was measured 48 h after infection. Representative data of three independent stellate cell isolations are shown with the mean of $n = 3$ per condition. * $P < 0.05$ for AdTL + CSRNLIDC-S11 vs AdTL. ** $P < 0.05$ for AdTL + CAR block vs AdTL - CAR block.

therapies is needed. In this study, we investigated whether selective adenoviral gene targeting to activated stellate cells is an option. We combined a PDGF β -receptor-specific peptide¹⁰ with a single-chain antibody fragment recognizing adenovirus type-5 fiber knob.^{14,15} In this way, we were able to construct a fusion protein (CSRNLIDC-S11) which targets

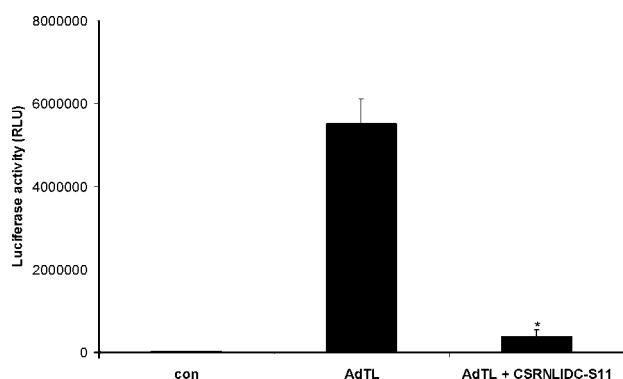


Figure 5. Untargeting of primary rat hepatocytes using PDGF-receptor β -targeted adenoviral gene transfer. Primary cultures of hepatocytes were infected with 50 pfu/cell after preincubation of AdTL virus with CSRNLIDC-S11 for 30 min. A ratio of 10 μ g of conjugate per 10^9 viral particles was used. Gene transfer is presented as luciferase activity (relative light units) measured 48 h after infection. Representative data of four independent hepatocyte isolations are shown with the mean of $n = 3$ per condition. * $P < 0.05$ for AdTL + CSRNLIDC S11 vs AdTL.

recombinant adenovirus type 5 specifically to activated stellate cells, whereas gene transfer in hepatocytes is dramatically reduced. Evidence for the specificity of our approach was provided by earlier competition studies on PDGF- $R\beta$ positive fibroblasts and activated stellate cells, using CSRNLIDC-peptide-modified albumin and PDGF-BB.¹⁰ We now demonstrate the feasibility of this approach in gene transfection. The present data demonstrate that the scrambled variant of the fusion protein did not mediate adenoviral gene transfer in NIH/3T3 fibroblasts. In contrast, the CSRNLIDC-peptide accomplished a 60-fold enhancement of the adenoviral-encoded luciferase expression. Moreover, in activated stellate cells, adenoviral-mediated gene transfer was enhanced by the CSRNLIDC-peptide and in a CAR-independent manner. Finally, we clearly demonstrated untargeting of PDGF- $R\beta$ -negative hepatocytes *in vitro*. These data indicate that PDGF- $R\beta$ -mediated gene transfer in stellate cells using CSRNLIDC-S11-modified recombinant virus is specific and more efficient than CAR-mediated infection. Recently, the involvement of heparan sulfate glycosaminoglycans in adenovirus type 5-host cell interactions was demonstrated.²⁸ These hydrophilic peptide strains are exposed on a variety of cell types. Although CAR-independent mechanisms exist for adenovirus entry, we have shown that they do not interfere with our targeting approach to the PDGF- $R\beta$: a clear selectivity was demonstrated between hepatocytes and activated stellate cells. Besides measuring luciferase expression, the AdTL virus allowed us to monitor GFP expression. The GFP observations are informative since they demonstrate that enhanced gene expression by PDGF β -

R-retargeted virus results from a higher number of transduced cells and not from a few cells expressing the genes more abundantly. The PelB leader in our construct allows the fusion protein to enter the bacterial periplasmic space where an S-S bridge can be formed and a cyclic CSRNLIDC peptide will be generated. We did not demonstrate this cyclic structure. However, a different fusion peptide without two cysteine amino acids in the PDGF- $R\beta$ -binding part (SRNLID-S11), hardly retargeted the adenovirus to NIH/3T3 fibroblasts (data not shown). These results confirm other data demonstrating that cyclic peptides display stronger binding activity to their receptor.²⁹ A few adenoviral gene therapy studies in cirrhotic rats were performed with nonmodified virus containing genes coding for hepatocyte growth factor, interferon α , or urokinase-type plasminogen activator.^{30–32} Attenuation of liver fibrosis through adenoviral delivery of matrix metalloproteinases was described as well.³³ Although fibrosis was reduced in these studies, normal liver histology was not completely restored because of the α -specificity of these unmodified vectors.¹⁷ In addition, a high amount of viral particles was needed to overcome low gene transfer in fibrotic livers and virus degradation in Kupffer cells.¹⁷ High amounts of virus are not favorable since mortality is higher among animals with fibrotic livers after receiving adenovirus compared to control animals.^{34,35} In addition, discrimination between activated and nonactivated stellate cells, endothelial cells, Kupffer cells, and hepatocytes was not accomplished in fibrotic livers.¹⁸ Furthermore, specific gene targeting allows reduced administration of adenoviral particles. This is of particular importance since in animals with liver fibrosis

(28) Dechecchi, M. C.; Melotti, P.; Bonizzato, A.; Santacatterina, M.; Chilosi, M.; Cabrini, G. Heparan sulfate glycosaminoglycans are receptors sufficient to mediate the initial binding of adenovirus types 2 and 5. *J. Virol.* **2001**, *75* (18), 8772–8780.

- (29) Koivunen, E.; Wang, B.; Ruoslahti, E. Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins. *Biotechnology (N.Y.)* **1995**, *13* (3), 265–270.
- (30) Salgado, S.; Garcia, J.; Vera, J.; Siller, F.; Bueno, M.; Miranda, A.; Segura, A.; Grijalva, G.; Segura, J.; Orozco, H.; Hernandez-Pando, R.; Fafutis, M.; Aguilar, L. K.; Guilar-Cordova, E.; Rmendariz-Borunda, J. Liver cirrhosis is reverted by urokinase-type plasminogen activator gene therapy. *Mol. Ther.* **2000**, *2* (6), 545–551.
- (31) Suzuki, K.; Aoki, K.; Ohnami, S.; Yoshida, K.; Kazui, T.; Kato, N.; Inoue, K.; Kohara, M.; Yoshida, T. Adenovirus-mediated gene transfer of interferon alpha improves dimethylnitrosamine-induced liver cirrhosis in rat model. *Gene Ther.* **2003**, *10* (9), 765–773.
- (32) Ueki, T.; Kaneda, Y.; Tsutsui, H.; Nakanishi, K.; Sawa, Y.; Morishita, R.; Matsumoto, K.; Nakamura, T.; Takahashi, H.; Okamoto, E.; Fujimoto, J. Hepatocyte growth factor gene therapy of liver cirrhosis in rats. *Nat. Med.* **1999**, *5* (2), 226–230.
- (33) Iimuro, Y.; Nishio, T.; Morimoto, T.; Nitta, T.; Stefanovic, B.; Choi, S. K.; Brenner, D. A.; Yamaoka, Y. Delivery of matrix metalloproteinase-1 attenuates established liver fibrosis in the rat. *Gastroenterology* **2003**, *124* (2), 445–458.
- (34) Smith, J. S.; Tian, J.; Muller, J.; Byrnes, A. P. Unexpected pulmonary uptake of adenovirus vectors in animals with chronic liver disease. *Gene Ther.* **2004**, *11* (5), 431–438.
- (35) Garcia-Banuelos, J.; Siller-Lopez, F.; Miranda, A.; Aguilar, L. K.; Guilar-Cordova, E.; Rmendariz-Borunda, J. Cirrhotic rat livers with extensive fibrosis can be safely transduced with clinical-grade adenoviral vectors. Evidence of cirrhosis reversion. *Gene Ther.* **2002**, *9* (2), 127–134.

unexpected pulmonary uptake of adenovirus was reported.³⁴ In addition, physical restraints for adenoviral transduction in liver fibrosis (absence of fenestrated endothelium, presence of fibrous matrix) may hamper specific delivery of adenovirus to hepatic stellate cells. Previously, it was demonstrated that in rats with liver fibrosis, CSRNLIDC-modified albumin quickly accumulated in the liver. Therefore, our PDGF-R β -directed adenovirus may improve the safety and efficacy of gene transfer to the activated stellate cell during liver fibrosis. Because the S11 antibody will shield the adenovirus knob, this approach also may result in untargeting of Kupffer cells. This selective gene transfer to activated stellate cells opens the possibility to induce apoptosis or to influence transcription-factors, like NF- κ B, in these cells. Both strategies are highly relevant for therapy^{5,7} but cannot be applied without activated stellate cell-selectivity since interference in other liver cells would be deleterious. A disadvantage of our two-component system, which consists of a fusion protein coupled to a virus, is that it might be difficult to transfer to a clinical setting. Therefore, it would be preferable to incorporate the targeting peptide into the adenoviral fiber knob. This has been performed successfully in previous studies.^{36–38} Whether insertion of the CSRNLIDC peptide in the adenoviral fiber knob also improves stellate cell-selective gene transfer remains to be established.

-
- (36) Michael, S. I.; Hong, J. S.; Curiel, D. T.; Engler, J. A. Addition of a short peptide ligand to the adenovirus fiber protein. *Gene Ther.* **1995**, *2* (9), 660–668.
- (37) Krasnykh, V.; Dmitriev, I.; Mikheeva, G.; Miller, C. R.; Belousova, N.; Curiel, D. T. Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J. Virol.* **1998**, *72* (3), 1844–1852.

Conclusion

Overall, our PDGF-R β -retargeted recombinant adenovirus allows selective gene transfer in activated stellate cells and prevents gene uptake by other cells like hepatocytes. Hepatocytes are generally responsible for most of the uptake of unmodified adenovirus, which seriously hampers efficiency of antifibrotic therapies based on adenoviral gene transfer. Using our new approach, a therapeutic gene can be selectively transferred in activated stellate cells, thereby sensitizing only these cells to apoptosis. The PDGF-R β -targeted adenovirus described in this paper is the first viral vector targeted to this important cell-type. This strategy may be beneficial for the treatment of fibrosis.

Acknowledgment. We are most grateful to Edwin Bremer (Department of Medical Biology, University Hospital Groningen, The Netherlands) for his assistance with FACS analysis and with the experimental setup. Furthermore, we are grateful to Manon Buist-Homan and Rick Havinga for their assistance with the isolation of hepatic stellate cells and hepatocytes (Department of Gastroenterology and Hepatology, Department of Pediatrics, University Hospital Groningen, The Netherlands). This work was supported by grants from the Groningen University Institute for Drug Exploration.

MP700118P

-
- (38) Wesseling, J. G.; Bosma, P. J.; Krasnykh, V.; Kashentseva, E. A.; Blackwell, J. L.; Reynolds, P. N.; Li, H.; Parameshwar, M.; Vickers, S. M.; Jaffee, E. M.; Huibregtse, K.; Curiel, D. T.; Dmitriev, I. Improved gene transfer efficiency to primary and established human pancreatic carcinoma target cells via epidermal growth factor receptor and integrin-targeted adenoviral vectors. *Gene Ther.* **2001**, *8* (13), 969–976.