CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Platelet-Derived Growth Factor (PDGF)-C Neutralization Reveals Differential Roles of PDGF Receptors in Liver and Kidney Fibrosis

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Platelet-derived growth factors (PDGF) are key mediators of organ fibrosis. We investigated whether *PDGF-C*^{-/-} mice or mice treated with neutralizing PDGF-C antibodies are protected from bile duct ligation-induced liver fibrosis, and we compared the effects with those of PDGF-C deficiency or neutralization on kidney fibrosis induced by unilateral ureteral obstruction. Unexpectedly, and in contrast to kidney fibrosis, PDGF-C deficiency or antagonism did not protect from liver fibrosis or functional liver impairment. Furthermore, the hepatic infiltration of monocytes/macrophages/dendritic cells and chemokine mRNA expression (CC chemokine ligand [CCL]5, CCL2, and CC chemokine receptor 2 [CCR2]) remained unchanged. Transcript expression of PDGF ligands increased in both liver and kidney fibrosis and was not affected by neutralization of PDGF-C. In kidney fibrosis, PDGF-C deficiency or antagonism led to reduced expression and signaling of PDGF-receptor (R)- α - and PDGFR- β -chains. In contrast, in liver fibrosis there was either no difference (*PDGF-C*^{-/-} mice) or even an upregulation of PDGFR- β and signaling (anti-PDGF-C group). Finally, *in vitro* studies in portal myofibroblasts pointed to a predominant role of PDGF-B and PDGF-D signaling in liver fibrosis. In conclusion, our study revealed significant differences between kidney and liver fibrosis in that PDGF-C mediates kidney fibrosis, whereas antagonism of PDGF-C in liver fibrosis appears to be counteracted by significant upregulation and increased PDGFR- β signaling. PDGF-C antagonism, therefore, may not be effective to treat liver fibrosis. (*Am J Pathol* 2013, 182: 107–117; <http://dx.doi.org/10.1016/j.ajpath.2012.09.006>)

Liver fibrosis, the common end-stage of chronic liver diseases, is characterized by an accumulation of extracellular matrix proteins and is associated with high morbidity and mortality. Cytokines and growth factors are critically involved in regulating liver cell activation, proliferation, and migration, as well as extracellular matrix production.¹ The PDGF family plays a key role in the pathogenesis of fibrotic diseases in the heart, lung, kidney, and liver.² PDGF-C acts via PDGFR- α , and it is a potent mitogen for fibroblasts and vascular smooth muscle cells *in vitro*.^{3,4} Observations in different organs suggest that PDGF-C plays an important role in the regulation of fibrosis. The first evidence of this

was derived from a mouse model in which transgenic over-expression of PDGF-C in the heart induced fibroblast proliferation resulting in cardiac fibrosis, hypertrophy, and ultimately cardiomyopathy.^{4,5} Transgenic mice with a lung-specific PDGF-C over-expression developed massive mesenchymal cell hyperplasia and died from respiratory insufficiency immediately after birth.⁶ Increased PDGF-C

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Table 1 Antibodies Used in this Study

Antibody	Cat No.	Supplier	Species	Dilution
PDGFR type- α	sc-338	Santa Cruz Biotechnology, Santa Cruz, CA	h, m, r	1:1000
PDGFR type- β	sc-958	Santa Cruz Biotechnology	h, m, r	1:1000
PDGFR type- β (ext. cellular domain)	PC17	Oncogene Research Products, Boston, MA	h, m, r	1:1000
P-Tyr clone 4G10	05-321	Millipore, Schwalbach, Germany	h, m, r	1:1000
Collagen type I	1310-01	Southern Biotechnology Associates, Birmingham, AL	h, m, r	1:400
F4/80	T-2017	BMA Dianova, Hamburg, Germany	h, m	1:1000
α -SMA	1184-1	Epitomics, Inc., Burlingame, CA	h, m, r	1:2000
β -Actin	A5441	Sigma, Taufkirchen, Germany	h, m, r	1:10,000
GAPDH	sc-32233	Santa Cruz Biotechnology	h, m, r	1:2000
P-PDGFR- α mAb (Y754, 1018)	9360	Cell Signaling Technology, Frankfurt, Germany	h, m, r	1:1000
P-PDGFR- β mAb (Y740, 751, 771, 1009, 1021)	9970	Cell Signaling Technology	h, m, r	1:1000
P-Akt	4051	Cell Signaling Technology	h, m, r	1:1000
Akt	2920	Cell Signaling Technology	h, m, r	1:1000
P-ERK1/2	9101	Cell Signaling Technology	h, m, r	1:1000
ERK1/2	9102	Cell Signaling Technology	h, m, r	1:1000

ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human; m, mouse; P-, phospho-; PDGFR, platelet-derived growth factor receptor; r, rat; SMA, smooth muscle actin.

expression has also been observed in different experimental models of organ fibrosis in heart and lung tissues.^{7,8}

We have detected an upregulated expression of PDGF-C in fibrotic interstitial areas in murine, rat, and human kidney fibrosis.^{9,10} Furthermore, specific inhibition of PDGF-C markedly reduced kidney fibrotic changes in experimental murine kidney fibrosis.¹¹ Using two independent

approaches for PDGF-C depletion in mice (ie, gene knockout, as well as a neutralizing antiserum), we observed virtually identical reductions of parameters of kidney fibrosis.¹¹

In contrast, little is known on the functional role of PDGF-C within the liver. PDGF-C transgenic mice with a liver-specific expression of the transgene develop liver

Table 2 Primers for Real-Time RT-PCR

Gene	Forward primer	Reverse primer	Probe
Primers for detection with SYBR Green			
<i>GAPDH</i>	5'-GGCAAATTCACGGCACAGT-3'	5'-AGATGGTGATGGGCTTCCC-3'	
<i>Col1a1</i>	5'-TCGCTTCACCTACAGCACCC-3'	5'-TGACTGTCTTGCCCCAAGTTC-3'	
<i>Col3a1</i>	5'-TGAAGATGTCGTTGATGTGCAG-3'	5'-GCAGTGGTATGTAATGTTCTGGGAG-3'	
<i>CCL2</i>	5'-TGGCTCAGCCAGATGCAGT-3'	5'-ATTGGGATCATCTTGCTGGTG-3'	
<i>CCL5</i>	5'-AGTGCTCCAATCTTGCACTCG-3'	5'-CACTTCTTCTCTGGGTTGGCA-3'	
<i>CCR2</i>	5'-TGTGATTGACAAGCACTTAGACCA-3'	5'-ATGACAGGATTAATGCAGCAGTGT-3'	
Primers for detection with Taqman probe			
<i>GAPDH</i>	5'-GGCAAATTCACGGCACAGT-3'	5'-AGATGGTGATGGGCTTCCC-3'	5'-AAGGCCGAGAATGGGAAGCTTG-TCATC-3'
<i>Fn1</i>	5'-GATGGAATCCGGGAGCTTTT-3'	5'-TGCAAGGCAACCACACTGAC-3'	5'-CCGGCCTGAGGCCCTGCAG-3'
<i>PDGF-A</i>	5'-ACTTCCTGATCTGGCCCCC-3'	5'-TGAAGGCTGGCACTTGACG-3'	5'-TGTGGAGGTGAAGCGCTGCAC-TG-3'
<i>PDGF-B</i>	5'-CCATCCGCTCCTTTGATGAT-3'	5'-AAGTCCAGCTCAGCCCCAT-3'	5'-CGCCTGCTGCACAGAGACTCC-GTA-3'
<i>PDGF-C</i>	5'-CTGGTGTGGAGATTAGTTGCAGTAG-3'	5'-CCAGCCCAAATCTCTCATCAA-3'	5'-TGAAAATGTGCGGATCCAGC-TGACA-3'
<i>PDGF-D</i>	5'-GACACTTTTGCGACTCCGC-3'	5'-TGTGAGGTGATTGCTCTCATCTC-3'	5'-TTGCGCAATGCCAACCTCA-GGA-3'
<i>PDGFR-α</i>	5'-AACGGAACCTTCAGCGTGG-3'	5'-AACTCGCTGGTCTTGAACGTC-3'	5'-CCTTACATCTGTGAGGCCACC-GTCAA-3'
<i>PDGFR-β</i>	5'-GAGGCTTATCCGATGCCTTCT-3'	5'-AACTAAGTTCGCCAGCGCC-3'	5'-CCTGTGGCTCAAGGACAACCG-TACCTT-3'

CCL2, CC chemokine ligand 2; CCL5, CC chemokine ligand 5; CCR2, CC chemokine receptor 2; Col1a1, collagen 1a1; Col3a1, Collagen 3a1; Fn1, fibronectin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PDGF, platelet-derived growth factor; PDGFR, PDGF-receptor.

fibrosis, steatosis, and hepatocellular carcinoma.¹² Therefore, we hypothesized that specific PDGF-C neutralization protects from liver fibrosis. We studied the potential profibrotic role of PDGF-C by using a neutralizing anti-PDGF-C antiserum in experimental murine liver fibrosis induced by bile duct ligation (BDL) and by inducing liver fibrosis in *PDGF-C*^{-/-} mice in the same model.

Materials and Methods

Anti-PDGF-C Antibody

The generation and specificity of neutralizing sheep anti-PDGF-C antibody and irrelevant sheep control IgG (kindly provided by Arne Östman, Karolinska Institute, Stockholm, Sweden) have been described.¹¹ Briefly, anti-PDGF-C antiserum was generated in sheep after immunization with recombinant core domain of PDGF-CC and was purified using a sheep IgG purification kit (Affiland, Ans, Belgium). Control irrelevant sheep IgG were purified from non-immunized sheep serum.

Animal Experiments

All studies were approved by the local Institutional Review Board. Animals were held in rooms with constant temperature and humidity, 12 hour light/12 hour darkness cycles, and had *ad libitum* access to drinking water (ozone-treated and acidified) and standard murine chow. Male C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). *PDGF-C*^{-/-} mice were provided by Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada) and Christer Betsholtz (Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden) and are described elsewhere.¹³ When bred in a mixed genetic background (between SV129 and C57BL/6), PDGF-C deficiency did not cause perinatal lethality. PDGF-C deficiency and/or wild-type (WT) status were confirmed by PCR.

Liver Fibrosis

All mice underwent an abdominal section and a double ligation of the common bile duct with 5-0 suture under anesthesia (inhalation of isoflurane) using a protocol that was adapted from rat surgery.^{14,15} In a first experiment, BDL was induced in 11-week-old *PDGF-C*^{-/-} mice (*n* = 5) and WT littermate controls (*n* = 8). In a second approach, BDL was induced in WT mice treated with neutralizing anti-PDGF-C antibody (10 mg/kg intraperitoneally) or equivalent amounts of control IgG (*n* = 8 per group). Antibodies were administered twice weekly for a total of two weeks at days 1, 3, 7, 10, and 14. Mice were sacrificed at day 14 in both experiments. At the time of sacrifice, serum was taken and liver tissue was harvested for histology and immunohistochemistry, as well as RNA and protein analyses.

Kidney Fibrosis

Unilateral ureteral obstruction (UVO) was induced in *PDGF-C*^{-/-} mice (*n* = 5) and littermate controls (*n* = 7), as well as in WT mice treated with neutralizing anti-PDGF-C antibody (*n* = 5) or control IgG (*n* = 5), as described.¹¹ Briefly, antibody treatment of WT mice started on day 1 and was continued until day 4 after UVO with single daily intraperitoneal injections of the compound (1 mg/mouse/day). Mice were sacrificed at day 5 after UVO and kidney tissue was obtained from the obstructed and nonobstructed kidney for (immune) histology, as well as RNA and protein analyses.

Figure 1 Quantification of BDL-induced liver fibrosis in *PDGF-C*^{+/+} or *PDGF-C*^{-/-} mice. **A:** Histological collagen deposition was analyzed by computer-assisted morphometry in Sirius Red-stained liver tissues from sham controls (*n* = 2), *PDGF-C*^{+/+} mice (*n* = 8), or *PDGF-C*^{-/-} mice (*n* = 5). Graphs show individual data from each mouse; bars indicate means. The images illustrate representative liver fibrosis (magnification, $\times 100$). **B:** Histological macrophage accumulation was analyzed by computer-assisted morphometry in F4/80-stained liver tissues (magnification, $\times 200$). **C:** Representative Western blot analyzed collagen type I (and GAPDH control) protein expression in total liver protein extracted from one sham control, four *PDGF-C*^{+/+}, and three *PDGF-C*^{-/-} mice. Bars indicate mean relative band intensities of both groups (*n* = 8 *PDGF-C*^{+/+}, *n* = 5 *PDGF-C*^{-/-}; dotted line indicates sham treated, nonfibrotic animals).

Figure 2 Quantification of liver mRNA expression BDL-induced liver fibrosis in *PDGF-C*^{+/+} or *PDGF-C*^{-/-} mice. Transcript expression of matrix components collagen type I (A), collagen type III (B), and fibronectin (C), as well as of inflammatory parameters CCL5 (D), CCL2 (E), and CCR2 (F) were analyzed in livers from *PDGF-C*^{+/+} mice (*n* = 8) or *PDGF-C*^{-/-} mice (*n* = 5). Dotted lines indicate sham treated, nonfibrotic animals.

Liver Morphology and Immunohistochemical Analysis

Tissues were fixed in methyl Carnoy’s solution. Three-micron paraffin sections were stained with Sirius Red. Immunohistochemical analyses were performed as described^{10,11} using the antibodies shown in Table 1. Negative controls for the immunohistochemical procedure consisted of substitution of the primary antibody with nonimmune IgG. For the quantification of positively stained areas 20 randomly selected periportal fields were photographed and evaluated by computer-based morphometry using the analysis version 3.1 software (Soft Imaging System GmbH; Münster, Germany). All analyses were performed in a blinded manner.

Western Blot Analyses

Frozen liver tissues were homogenized with a tight-fitting Dounce homogenizer in 0.6 mL protein extraction buffer (50 mmol/L Hepes, pH 7.5, 150 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, 1% Triton X-100, pH 7.0) implemented with a protein and phosphatase inhibitor cocktail (1%; Sigma-Aldrich, Taufkirchen, Germany). After incubation on ice for 5 minutes, the samples were subjected to ultrasound treatment (3 × 10 seconds) and centrifuged for 5 minutes at 13,000 rpm. The supernatant was taken and protein content was measured in a bicinchoninic acid protein assay, according to the manufacturer’s instructions (Uptima, Montlucon Cedex, France).

For Western blot analyses 40 µg of whole protein lysates were loaded in each lane and separated on a 4% to 12%

NuPAGE gradient gel (Invitrogen, Karlsruhe, Germany). Proteins were transferred to nitrocellulose membranes (pore size, 0.45 µm; Schleicher and Schuell, Keene, NH) and probed with the antibodies listed in Table 1. The primary antibodies were detected with horseradish peroxidase-conjugated anti-goat or anti-rabbit antibodies and were visualized with the Super-Signal chemiluminescent substrate (Pierce Thermo Scientific, Rockford, IL) after extensive washing of the membranes. The

Table 3 Serum Parameters of *PDGF-C*^{+/+} and *PDGF-C*^{-/-} Mice 14 Days after Bile Duct Ligation

Parameter	<i>PDGF-C</i> ^{+/+} BDL* (<i>n</i> = 8)	<i>PDGF-C</i> ^{-/-} BDL* (<i>n</i> = 5)	Control (<i>n</i> = 2) [†]
Bilirubin (total) (mg/dL)	15.3 ± 5.3	13.2 ± 4.8	<0.1/<0.1
Bilirubin (direct) (mg/dL)	15.1 ± 5.2	12.8 ± 4.8	<0.1/<0.1
Protein (g/L)	39.5 ± 6.7	42.0 ± 10.4	40/42
Albumin (g/L)	25.8 ± 4.2	27.0 ± 5.1	20.6/29.6
Triglycerides (mg/dL)	69.3 ± 20.8	78.0 ± 17.5	62/64
Cholesterol (mg/dL)	481 ± 221	517 ± 317	64/54
HDL (mg/dL)	8.13 ± 7.24	5.2 ± 4.92	48/44
LDL (mg/dL)	18.6 ± 13.9	14.2 ± 11.6	10/<3
AST (U/L)	876 ± 990	863 ± 620	126/48
ALT (U/L)	630 ± 827	504 ± 256	78/24
AP (U/L)	416 ± 537	568 ± 796	4/8

*There were no significant differences between both BDL groups (*t*-test).

[†]Values for each animal (*n* = 2) are shown.

ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate transaminase; BDL, bile duct ligation; control, non-BDL sham-treated *PDGF-C*^{+/+} littermates; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

intensity of the signals was quantified using the Quantity One software 4.6 Basic (BIO-RAD, Munich, Germany).

Immunoprecipitation

For immunoprecipitation of PDGFR- α , 1 mg of tissue lysates were pre-cleared with protein G agarose for 1 hour, and the supernatants were incubated with 1 μ g of PDGFR- α antibody overnight, followed by protein agarose precipitation. After washing, the precipitated samples were solubilized in lithium dodecyl sulfate sample buffer, with dithiothreitol as a reducing reagent and the resulting supernatant separated in 4% to 12% Bis-Tris gel and immunoblotted onto the nitrocellulose membranes. Membranes were probed for phospho-Tyr or phospho-PDGFR- α and for total PDGFR- α to confirm the immunoprecipitation efficiency. All antibodies used are listed in Table 1.

Quantitative Real-Time PCR

Total RNA was extracted from liver and kidney tissue and cDNA syntheses, and real-time quantitative PCRs were performed as previously described.¹⁶ TaqMan primers were designed from sequences in the GenBank database using Primer Express software for Real-Time PCR, version 3.0 (Applied Biosystems, Darmstadt, Germany). The sequences used in this study are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification was used as an internal standard.

Portal Fibroblast Cell Culture Experiments

Portal fibroblasts were isolated from male Sprague-Dawley rats (500 to 750 g), as described.^{17,18} To assess portal myofibroblast proliferation induced by PDGF ligands and transforming growth factor- β 1, cells were seeded at 50,000 cells per well in 12-well cultured plates using Dulbecco's modified Eagle's medium containing 10% fetal calf serum. On 70% confluence, cells were starved in 0.5% fetal calf serum for 24 hours and subsequently stimulated by adding 25 ng/mL of 1 of 4 PDGF isoforms or 1 ng/mL transforming growth factor- β 1 (R&D Systems, Wiesbaden, Germany). Cells were labeled with 10 μ mol/L 5-bromo-2'-deoxyuridine (BrdU) for 24 hours using the Cell ELISA BrdU colorimetric assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. For Western blot analyses of lysates from stimulated portal myofibroblast cell lysates were prepared in radio-immunoprecipitation assay buffer containing 20 mmol/L Tris-HCl (pH 7.2), 150 mmol/L NaCl, 2% (w/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, and the Complete Protease Inhibitors solution (Roche Diagnostics). Equal amounts (20 μ g/lane) of total protein were then separated under reducing conditions in 4% to 12% Bis-Tris gradient gels using NuPAGE MOPS or NuPAGE MES running buffer (all Invitrogen reagents). Separated proteins

were electroblotted onto nitrocellulose membranes and specific proteins were detected as previously outlined.

Serum Measurements

Total and direct bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase total protein, HDL, LDL, cholesterol, and triglycerides were measured on a Modular Pre-Analytics system (Roche Diagnostics).

Statistical Analysis

All results are presented as means $\pm$ SD. Statistical significance (defined as $P < 0.05$) was evaluated using the unpaired Student's *t*-test.

Figure 3 Quantification of BDL-induced liver fibrosis in wild-type mice treated with control IgG or neutralizing anti-PDGF-C antibody. **A:** Histological collagen deposition was analyzed by computer-assisted morphometry in Sirius Red-stained liver tissues from mice treated with control IgG ($n = 8$) or mice treated with neutralizing anti-PDGF-C antibody ($n = 8$). Graphs show individual data from each mouse; bars indicate means. Images illustrate representative liver fibrosis (magnification, $\times 100$). **B:** Histological macrophage accumulation was analyzed by computer-assisted morphometry in F4/80-stained liver tissues (magnification, $\times 200$). **C:** Representative Western blot analyzed collagen type I (and GAPDH control) protein expression in total liver protein extracted from one sham control, four mice treated with control IgG, and four mice treated with neutralizing anti-PDGF-C antibody. Bars indicate mean relative band intensities of both groups ($n = 8$ PDGF-C^{+/+}, $n = 8$ PDGF-C^{-/-}; dotted line indicates sham-treated, nonfibrotic animals).

Figure 4 Quantification of liver mRNA expression after BDL-induced liver fibrosis in mice treated with control IgG or neutralizing anti-PDGF-C antibody. Transcript expression of matrix components collagen type I (A), collagen type III (B), and fibronectin (C) as well as of inflammatory parameters CCL5 (D), CCL2 (E), and CCR2 (F) was analyzed in livers from mice treated with control IgG ($n = 8$) or neutralizing anti-PDGF-C antibody ($n = 8$). Dotted lines indicate sham-treated, non-fibrotic animals.

Results

PDGF-C Deficiency or Treatment With a Neutralizing Anti-PDGF-C Antibody Does Not Protect From Murine Bile Duct Ligation-induced Liver Fibrosis

Liver fibrosis was first induced in *PDGF-C*^{-/-} mice and in WT littermates. Fourteen days after bile duct ligation, significant liver fibrosis was observed, paralleled by an

Table 4 Serum Parameters of Control IgG and Anti-PDGF-C-Treated Mice 14 Days after Bile Duct Ligation

Parameter	Control IgG BDL* ($n = 8$)	Anti-PDGF-C BDL* ($n = 8$)	Control ($n = 3$)
Bilirubin (total) (mg/dL)	24.8 ± 4.0	23.9 ± 2.6	1.0 ± 0.0
Bilirubin (direct) (mg/dL)	24.0 ± 3.1	22.5 ± 2.0	1.0 ± 0.0
Protein (g/L)	46.3 ± 2.4	47.6 ± 3.1	ND
Albumin (g/L)	34.1 ± 1.3	34.5 ± 1.9	16.7 ± 1.2
Triglycerides (mg/dL)	109 ± 14	98 ± 14	n.m.
Cholesterol (mg/dL)	505 ± 248	434 ± 155	10.0 ± 10.0
HDL (mg/dL)	17.5 ± 3.3	18.3 ± 3.5	33.3 ± 5.8
LDL (mg/dL)	48.3 ± 9.9	58.3 ± 17.2	30.0 ± 0.0
AST (U/L)	705 ± 290	784 ± 272	93.3 ± 23.1
ALT (U/L)	549 ± 170	581 ± 173	40 ± 0.0
AP (U/L)	1049 ± 181	1014 ± 129	5.0 ± 0.0

*There were no significant differences between both BDL groups (t -test). ALT, alanine transaminase; AP, alkaline phosphatase; AST, aspartate transaminase; BDL, bile duct ligation; control, non-BDL, sham-treated C57BL6; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ND, not determined.

increase in inflammatory parameters. Compared to WT littermates, *PDGF-C*^{-/-} mice exhibited the same degree of Sirius Red-positive fibrotic areas (Figure 1A), collagen type I protein accumulation measured by Western blot (Figure 1C), and of collagen type I, collagen type III, and fibronectin mRNA expression (Figure 2, A–C). Functional liver injury, as indicated by various serum parameters, did not differ between the two groups (Table 3). In a second approach, liver fibrosis was induced in WT mice, which were treated with either neutralizing anti-PDGF-C antibody or irrelevant IgG control. The results were nearly identical to the first approach, in that no differences evolved between the livers of controls and PDGF-C-depleted mice (Figure 3, A and C; Figure 4, B and C; Table 4). One exception was collagen type I mRNA expression, which increased in the livers of anti-PDGF-C antibody-treated animals (Figure 4A). Inflammatory parameters (ie, CCL2-mRNA, CCL5-mRNA, or CC chemokine receptor 2 (CCR2)-mRNA expression, or hepatic infiltration of F4/80-positive macrophages) increased in BDL. However, no difference was found between *PDGF-C*^{-/-} mice and WT littermates (Figure 1B and Figure 2, D–F), or between mice treated with anti-PDGF-C antibody and control IgG-treated animals (Figure 3B and Figure 4, D–F).

Differences in PDGFR Expression and Activation between Experimental Murine Liver and Kidney Fibrosis

We have previously used the same *PDGF-C*^{-/-} mice and the same neutralizing PDGF-C antibody in a murine kidney fibrosis model, and we have been able to demonstrate

Figure 5 Quantification of hepatic and renal PDGFR- α and PDGFR- β mRNA expression in experimental liver and kidney fibrosis. PDGFR- α and PDGFR- β mRNA expression was analyzed in liver tissue after BDL in *PDGF-C*^{+/+} ($n = 8$) and *PDGF-C*^{-/-} mice ($n = 5$) (A), and [in mice treated with control IgG ($n = 8$) or neutralizing anti-PDGF-C antibody ($n = 8$) (B)]. PDGFR mRNA expression was analyzed in kidney tissue after unilateral ureteral obstruction in *PDGF-C*^{+/+} ($n = 7$) and *PDGF-C*^{-/-} mice ($n = 5$) (C), and in mice treated with control IgG ($n = 5$) or neutralizing anti-PDGF-C antibody ($n = 5$) (D).

significant anti-fibrotic effects with these approaches.¹¹ To assess why PDGF-C deficiency or antagonism is beneficial in the kidney, but not with liver fibrosis, we next analyzed the intrahepatic versus renal expression of the PDGFR system.

PDGFR- α and PDGFR- β mRNA expressions were not different in livers from *PDGF-C*^{-/-} mice compared to *PDGF-C*^{+/+} mice at day 14 after BDL (Figure 5A). For PDGFR- α , this expression was confirmed on the protein level (Figure 6A and 6B), whereas protein expression of PDGFR- β tended to be (nonsignificantly) increased in *PDGF-C*^{-/-} mice (Figure 6A and 6B). Western blotting for activated receptors revealed no differences for phosphorylated PDGFR- α (Figure 6A and 6B), but showed a significant

increase for phosphorylated PDGFR- β in *PDGF-C*^{-/-} mice compared to WT littermates (Figures 6A and 6B). After treatment with neutralizing anti-PDGF-C antibody, as a second approach in BDL, we detected a significant increase of PDGFR- β mRNA expression compared to treatment with irrelevant control IgG, although there was no difference in PDGFR- α expression between these two groups (Figure 5B). In summary, these data are indicative of an upregulation of PDGFR- β expression and activation on neutralization of PDGF-C in BDL-induced liver fibrosis, with no changes of PDGFR- α .

In experimental kidney fibrosis induced by UUO, kidneys from *PDGF-C*^{-/-} mice showed a nonsignificant trend

Figure 6 Hepatic expression and activation of PDGFR in *PDGF-C*^{+/+} and *PDGF-C*^{-/-} mice after BDL. The hepatic expression of total and phosphorylated (activated) PDGFR- α and PDGFR- β was analyzed in liver tissues from *PDGF-C*^{+/+} mice and *PDGF-C*^{-/-} mice 14 days after BDL. A: SDS-PAGE and Western blot analyses with specific antibodies. PDGFR- α was immunoprecipitated (IP) before detection to increase the sensitivity. B: Quantification of the Western blot bands by densitometry. The GAPDH signal was used as a loading control and for normalization.

Figure 7 Renal expression and activation of PDGFR in *PDGF-C*^{+/+} and *PDGF-C*^{-/-} mice after unilateral ureteral obstruction. The renal expression of total and phosphorylated, activated PDGFR- α and PDGFR- β was analyzed in kidney tissues from *PDGF-C*^{+/+} mice and *PDGF-C*^{-/-} mice 5 days after unilateral ureteral obstruction. A: SDS-PAGE and Western blot analyses with specific antibodies. PDGFR- α was immunoprecipitated (IP) before detection to increase the sensitivity. B: Quantification of the Western blot bands by densitometry. The GAPDH signal was used as a charge control and for normalization. CL, contralateral, non-fibrotic kidneys.

Figure 8 Quantification of hepatic and renal platelet-derived growth factor (PDGF) ligand mRNA expression in experimental liver and kidney fibrosis. PDGF-A, PDGF-B, PDGF-C, and PDGF-D mRNA expression was analyzed in liver tissue after bile duct ligation in *PDGF-C*^{+/+} (*n* = 8) and *PDGF-C*^{-/-} mice (*n* = 5) (A), and in mice treated with control IgG (*n* = 8) or neutralizing anti-PDGF-C antibody (*n* = 8) (B). PDGF-A, PDGF-B, PDGF-C, and PDGF-D mRNA expression was analyzed in kidney tissue after unilateral ureteral obstruction in *PDGF-C*^{+/+} (*n* = 7) and *PDGF-C*^{-/-} mice (*n* = 5) (C), and in mice treated with control IgG (*n* = 5) or neutralizing anti-PDGF-C antibody (*n* = 5) (D). Dotted lines indicate sham-treated animals (A, B) or contralateral controls (C, D).

toward decreased PDGFR- α and PDGFR- β mRNA expression in comparison to WT littermates on day 5 after disease induction (Figure 5C). Western blotting demonstrated a marked increase of renal protein expression of both PDGF receptors during UUU compared to contralateral controls (Figure 7, A and B). Renal PDGFR- β protein expression was not different between *PDGF-C*^{-/-} mice and their WT

littermates, whereas PDGFR- α expression was significantly reduced in *PDGF-C*^{-/-} mice (Figure 7, A and B). Demonstrated by the detection of phosphorylated PDGFR- α and PDGFR- β , both receptors were highly activated during UUU and this was significantly reduced in *PDGF-C*^{-/-} mice (Figure 7, A and B). By using anti-PDGF-C antibody treatment as a second PDGF-C depleting approach in

experimental kidney fibrosis, we detected a significant reduction of both PDGFR- α and PDGFR- β mRNA (Figure 5D). Collectively, these data indicate a down-regulation of PDGFR- α expression and activation, as well as PDGFR- β activation on neutralization of PDGF-C in UUO-induced kidney fibrosis.

Expression of PDGF Ligands Does Not Differ between *PDGF-C*^{-/-} Mice or PDGF-C Neutralized Mice and Their Controls

Altered expression of PDGF ligands could also have accounted for the differential activation of PDGFRs and the significant reduction of fibrosis in the kidney, but not in the liver. We, therefore, measured transcript levels of PDGF-A, PDGF-B, PDGF-C, and PDGF-D in fibrotic livers and kidneys of both *PDGF-C*^{-/-} mice and anti-PDGF-C antibody-treated mice compared to their respective controls. As shown (in Figure 8, A and B), in liver fibrosis, PDGF-ligand transcript expression was increased compared to non-diseased controls. Neither PDGF-C deficiency nor antagonism led to differential expression of any PDGF ligand. Expectably, PDGF-C mRNA could not be detected in PDGF-C-deficient mice (Figure 8A). In fibrotic kidneys, PDGF ligand transcript expression was increased compared to contralateral kidneys and unchanged in PDGF-C-deficient or PDGF-C-neutralized mice (Figure 8, C and D). However, in contrast to the liver, there were slight reductions of PDGF ligand expression on PDGF-C depletion in the kidney. Renal PDGF-C mRNA could not be detected in *PDGF-C*^{-/-} mice (Figure 8C).

PDGF Isoform Signaling in Portal Myofibroblasts

Portal myofibroblasts and bile duct epithelia in the liver proliferate markedly in the first 48 hours on BDL. Because portal myofibroblasts play an important role in early portal

fibrosis, we first analyzed the expression of PDGFRs by these cells and investigated PDGF ligand-induced signaling and proliferation. Both receptors were expressed by these cells, but only stimulation with PDGF-B and PDGF-D resulted in significant signaling, as evidenced by PDGFR-Tyr phosphorylation, ERK1/2-activation, and Akt-activation (Figure 9A). In addition, all PDGF isoforms significantly induced portal myofibroblast proliferation, but PDGF-B and PDGF-D showed the strongest effects (Figure 9B).

Discussion

The major novel finding of the present study is the observation that specific inhibition of PDGF-C did not reduce experimental liver fibrosis in the murine BDL model. Using two independent approaches for PDGF-C depletion in mice (ie, gene knockout and a neutralizing antibody), we observed no differences in liver fibrosis and inflammation. These findings are in clear contrast with our own findings in experimental kidney fibrosis using the same reagents and mice.¹¹ In the kidney, specific PDGF-C neutralization significantly reduced the development of unilateral ureteral obstruction induced fibrosis and inflammation.¹¹

To analyze the mechanism(s) responsible for this surprising difference between the organs, as the second major finding of this study, we identified a differential regulation of PDGFR expression and signaling. PDGFR expression and signaling remained at a high level in fibrotic PDGF-C-depleted livers or even showed upregulation and enhanced signaling in the case of PDGFR- β on treatment with anti-PDGF-C antibody. In contrast, both expression and signaling of PDGFR- α and PDGFR- β were reduced in fibrotic kidneys of PDGF-C-deficient or anti-PDGF-C antibody-treated mice. This difference in PDGF receptor regulation and activation obviously accounted for the major organ difference with regard to fibrosis and inflammation on depletion or antagonism of PDGF-C. Another explanation

Figure 9 Platelet-derived growth factor (PDGF) isoform signaling in cultured portal myofibroblasts (pMF). **A:** SDS-PAGE and Western blot of pMF after 20 minutes of either no stimulation (first lane) or stimulation with PDGF isoforms (lanes 2–5) demonstrating PDGF-B and PDGF-D to be the most prominent activators of PDGFR-Tyr autophosphorylation (p-Tyr) and of downstream signaling molecules phospho-ERK1/2 (p-ERK1/2), and phospho-Akt (p-Akt). **B:** 5-bromo-2'-deoxyuridine proliferation assay. All PDGF isoforms significantly induced pMF proliferation, but PDGF-B and PDGF-D showed the strongest effects. By contrast, transforming growth factor- β efficiently inhibited pMF proliferation. The experiments were independently performed three times with triplicate measurements. OD, optical density.

could have been a differential, organ-specific expression of PDGF ligands. We failed, however, to detect a significant difference of PDGF ligand transcript expression in the fibrotic liver and fibrotic kidney on PDGF-C deficiency or PDGF-C neutralization. It cannot be completely excluded that there is a differential expression of the individual PDGF ligands on the protein level. In addition, during fibrosis, locally changed proteolytic activities on latent PDGF-C or PDGF-D might also play a role that can lead to reduced binding to and activation of PDGF receptors in the kidney, but not in the liver.

Is it unexpected that we were unable to identify an anti-fibrotic effect of PDGF-C neutralization in experimental liver fibrosis? Hepatic transcripts of PDGF ligands and receptors were upregulated on BDL-induced liver fibrosis, but with higher upregulation of PDGF-B, PDGF-D, and receptor- β than PDGF-A, PDGF-C, and PDGFR- α .¹⁹ Furthermore, primary hepatic stellate cells or myofibroblasts showed a significant proliferation only when stimulated with PDGF-B or PDGF-D, but not with PDGF-A or PDGF-C.¹⁹ This is in contrast to renal myofibroblasts, which have been demonstrated to be potently activated by PDGF-C.¹¹ Also, during renal fibrosis, leukocyte-derived PDGF-C may induce chemokine expression by fibroblasts, which may lead to the recruitment of additional leukocytes, creating an amplification loop for renal inflammation and fibrosis.¹¹ Accordingly, PDGF-C deficiency significantly reduced renal leukocyte infiltration¹¹; whereas, during liver fibrosis in the present study, PDGF-C deficiency had no significant effect on the infiltration of leukocytes in the liver. We also investigated portal myofibroblasts, which play an important role in liver scar formation, especially in biliary fibrosis.²⁰ In contrast to PDGF-A and PDGF-C, PDGF-B and PDGF-D emerged as potent inducers of PDGFR signaling and cell proliferation. Consistent with the previously described data, suggesting PDGFR- β signaling to be more important than PDGFR- α signaling in liver fibrosis, a recent study demonstrated that BDL-induced murine liver fibrosis was less severe in mice treated with an anti-PDGF-B antibody.²¹ Furthermore, liver fibrosis has also been induced in mice by liver-specific over-expression of PDGF-B.²² Collectively, in the liver, these data point to a stronger role of PDGF-B and PDGF-D, and PDGFR- β signaling in the formation of liver fibrosis than signaling via PDGF-C and PDGFR- α .

The individual contributions of PDGFR- α and PDGFR- β signaling in the development of liver fibrosis are still incompletely understood. The present study is the first to report specific PDGF-C antagonism in experimental liver fibrosis *in vivo*. The lack of any therapeutic effect of an anti-PDGF-C treatment (in two independent and complementary experimental settings) together with the documented benefit of an anti-PDGF-B treatment indicates that PDGFR- β signaling might be more important in the pathogenesis of liver fibrosis. The different role of PDGF-C in the development of kidney fibrosis is striking, and the lack of an

up-regulation of renal PDGFR- β expression in response to anti-PDGF-C treatment might explain the significant differences of anti-PDGF-C treatment in kidney or liver fibrosis.

In conclusion, specific PDGF-C antagonism did not protect from BDL-induced murine liver fibrosis. PDGF-receptor expression analyses identified a significant up-regulation of the PDGFR- β in response to anti-PDGF-C treatment that might have initiated a PDGFR- β -driven escape mechanism. PDGF-C antagonism, therefore, may not be a promising treatment of liver fibrosis.

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References

- Hernandez-Gea V, Friedman SL: Pathogenesis of liver fibrosis. *Annu Rev Pathol* 2011, 6:425–456
- Bonner JC: Regulation of PDGF and its receptors in fibrotic diseases. *Cytokine Growth Factor Rev* 2004, 15:255–273
- 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, 276:27406–27414
- 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, 2:302–309
- Ponten A, Li X, Thoren P, Aase K, Sjoblom T, Ostman A, Eriksson U: Transgenic overexpression of platelet-derived growth factor-C in the mouse heart induces cardiac fibrosis, hypertrophy, and dilated cardiomyopathy. *Am J Pathol* 2003, 163:673–682
- Zhuo Y, Hoyle GW, Shan B, Levy DR, Lasky JA: Over-expression of PDGF-C using a lung specific promoter results in abnormal lung development. *Transgenic Res* 2006, 15:543–555
- Grun K, Markova B, Bohmer FD, Berndt A, Kosmehl H, Leipner C: Elevated expression of PDGF-C in coxsackievirus B3-induced chronic myocarditis. *Eur Heart J* 2005, 26:728–739
- Zhuo Y, Zhang J, Laboy M, Lasky JA: Modulation of PDGF-C and PDGF-D expression during bleomycin-induced lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2004, 286:L182–L188
- Eitner F, Ostendorf T, Kretzler M, Cohen CD, Eriksson U, Grone HJ, Floege J: PDGF-C expression in the developing and normal adult human kidney and in glomerular diseases. *J Am Soc Nephrol* 2003, 14: 1145–1153
- Eitner F, Ostendorf T, Van Roeyen C, Kitahara M, Li X, Aase K, Grone HJ, Eriksson U, Floege J: Expression of a novel PDGF isoform. PDGF-C, in normal and diseased rat kidney. *J Am Soc Nephrol* 2002, 13:910–917
- Eitner F, Bucher E, van Roeyen C, Kunter U, Rong S, Seikrit C, Villa L, Boor P, Fredriksson L, Backstrom G, Eriksson U, Ostman A, Floege J, Ostendorf T: PDGF-C is a proinflammatory cytokine that mediates renal interstitial fibrosis. *J Am Soc Nephrol* 2008, 19: 281–289
- Campbell JS, Hughes SD, Gilbertson DG, Palmer TE, Holdren MS, Haran AC, Odell MM, Bauer RL, Ren HP, Haugen HS, Yeh MM,

- Fausto N: Platelet-derived growth factor C induces liver fibrosis, steatosis, and hepatocellular carcinoma. *Proc Natl Acad Sci USA* 2005, 102:3389–3394
13. Ding H, Wu X, Bostrom H, Kim I, Wong N, Tsoi B, O'Rourke M, Koh GY, Soriano P, Betsholtz C, Hart TC, Marazita ML, Field LL, Tam PP, Nagy A: A specific requirement for PDGF-C in palate formation and PDGFR- α signaling. *Nat Genet* 2004, 36: 1111–1116
 14. Arias M, Sauer-Lehnen S, Treptau J, Janoschek N, Theuerkauf I, Buettner R, Gressner AM, Weiskirchen R: Adenoviral expression of a transforming growth factor- β 1 antisense mRNA is effective in preventing liver fibrosis in bile-duct ligated rats. *BMC Gastroenterol* 2003, 3:29
 15. Kountouras J, Billing BH, Scheuer PJ: Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. *Br J Exp Pathol* 1984, 65:305–311
 16. Ostendorf T, van Roeyen CR, Peterson JD, Kunter U, Eitner F, Hamad AJ, Chan G, Jia XC, Macaluso J, Gazit-Bornstein G, Keyt BA, Lichenstein HS, LaRochelle WJ, Floege J: A fully human monoclonal antibody (CR002) identifies PDGF-D as a novel mediator of mesangioproliferative glomerulonephritis. *J Am Soc Nephrol* 2003, 14: 2237–2247
 17. Kruglov EA, Jain D, Dranoff JA: Isolation of primary rat liver fibroblasts. *J Investig Med* 2002, 50:179–184
 18. Meurer SK, Tihaa L, Borkham-Kamphorst E, Weiskirchen R: Expression and functional analysis of endoglin in isolated liver cells and its involvement in fibrogenic Smad signalling. *Cell Signal* 2011, 23:683–699
 19. Borkham-Kamphorst E, van Roeyen CR, Ostendorf T, Floege J, Gressner AM, Weiskirchen R: Pro-fibrogenic potential of PDGF-D in liver fibrosis. *J Hepatol* 2007, 46:1064–1074
 20. Beaussier M, Wendum D, Schiffer E, Dumont S, Rey C, Lienhart A, Housset C: Prominent contribution of portal mesenchymal cells to liver fibrosis in ischemic and obstructive cholestatic injuries. *Lab Invest* 2007, 87:292–303
 21. Ogawa S, Ochi T, Shimada H, Inagaki K, Fujita I, Nii A, Moffat MA, Katragadda M, Violand BN, Arch RH, Masferrer JL: Anti-PDGF-B monoclonal antibody reduces liver fibrosis development. *Hepatol Res* 2010, 40:1128–1141
 22. Czochra P, Klopčič B, Meyer E, Herkel J, Garcia-Lazaro JF, Thieringer F, Schirmacher P, Biesterfeld S, Galle PR, Lohse AW, Kanzler S: Liver fibrosis induced by hepatic overexpression of PDGF-B in transgenic mice. *J Hepatol* 2006, 45:419–428