Expression of a Novel PDGF Isoform, PDGF-C, in Normal and Diseased Rat Kidney

FRANK EITNER,* TAMMO OSTENDORF,* CLAUDIA VAN ROEYEN,* MASASHI KITAHARA,* XURI LI,† KARIN AASE,† HERMANN-JOSEF GRÖNE,‡ ULF ERIKSSON,§ and JÜRGEN FLOEGE* 

*Division of Nephrology and Immunology, University of Aachen, Aachen, Germany; †Ludwig Institute for Cancer Research, Stockholm, Sweden; and ‡German Cancer Research Institute, DKFZ Heidelberg, Germany.

Abstract. Platelet-derived growth factor–C (PDGF-C) is a new member of the PDGF family. Its expression in normal and diseased kidney is unknown. Rabbit antisera were generated against human full-length, core domain, and mouse PDGF-C, and their specificity was confirmed by Western blot analyses. Renal PDGF-C expression was analyzed by immunohistochemistry in normal rats (n = 8), mesangioproliferative anti-Thy 1.1 nephritis (n = 4 each at days 1, 4, 6, and 85), passive Heymann nephritis (PHN, n = 4), puromycin nephrosis (PAN, n = 2), Milan normotensive rats (MN, n = 2), and obese Zucker rats (n = 3). PDGF-C expression was also studied in anti-Thy 1.1 rats treated with PDGF-B aptamer antagonists (n = 5) or irrelevant control aptamers (n = 5). PDGF-C was constitutively expressed in arterial smooth muscle cells and collecting duct epithelial cells. Mesangial PDGF-C was markedly upregulated in anti-Thy 1.1 nephritis in parallel with the peak mesangial cell proliferation. Furthermore, PDGF-CC acted as a potent growth factor for mesangial cells in vitro. Inhibition of PDGF-B via specific aptamers reduced the injury in anti-Thy 1.1 nephritis but did not affect the glomerular PDGF-C overexpression or the mitogenicity of PDGF-CC in vitro. In PHN, PAN, and obese Zucker rats, glomerular remaining negative for PDGF-C despite severe glomerular injury. The use of different antisera resulted in virtually identical findings. It is concluded that PDGF-C is a novel mesangial cell mitogen that is constitutively expressed in the kidney and specifically upregulated in mesangial, visceral epithelial, and interstitial cells after predominant injury to these cells. PDGF-C may therefore be involved in the pathogenesis of renal scarring.

Members of the platelet-derived growth factor (PDGF) family of cytokines are important mitogens and chemotactants for many types of mesenchymal cells (1–3). Until recently, the PDGF family comprised three dimers composed of a PDGF A- and B-chain, i.e., PDGF-AA, -AB, and -BB (4). It is well established by now that these PDGF are involved in different aspects of renal disease, in particular the mediation of glomerular mesangial cell proliferation and the induction of renal interstitial fibrosis (5,6). Both known subunits of the PDGF receptor, i.e., the α-subunit and β-subunit are constitutively expressed in the kidney. Although the α-subunit is present in vascular smooth muscle cells and the renal interstitium, the β-subunit is constitutively expressed in mesangial and parietal glomerular epithelial cells, in vascular smooth muscle cells, and in renal interstitial cells (4). Increased expression of PDGF receptors at sites of renal injury has been documented in a large variety of diseases (4).

A new member of the PDGF family has recently been identified and subsequently termed PDGF-C (7). PDGF-C, like PDGF-A and -B, forms a disulphide-bonded dimer, PDGF-CC. Li et al. (7) identified PDGF-CC as a PDGFR-α-specific ligand, and Gilbertsson et al. (8) showed that PDGF-CC could activate the beta receptor in a heterodimeric complex. There is no detectable binding or activation to beta receptor homodimers (Gilbertsson et al. (8) and our unpublished data). Transgenic overexpression of PDGF-C in the heart induced a significant proliferation of myocardial interstitial cells in addition to an increase in extracellular matrix production (7). To date, information on PDGF-C in the kidney is limited to the demonstration of abundant PDGF-C transcripts in human kidney (7). In addition, strong PDGF-C mRNA expression was detected in the metanephric mesenchyme aggregates during murine nephrogenesis, suggesting a role for PDGF-C in mesenchymal epithelial conversion as a prelude to tubular development (7). This study is the first to identify PDGF-C as a potent mitogenic stimulus for cultured mesangial cells in vitro. The localization of the cytokine PDGF-C in normal or diseased adult renal tissues as well as its function in vivo are unknown. We therefore analyzed the expression of PDGF-C by immunohistochemistry in renal tissues obtained from healthy normal adult rats and from rats with different renal diseases. Studied renal diseases included rats with immune-mediated mesangiproliferative anti-Thy 1.1 glomerulonephritis, rats with im-
mune-mediated podocyte injury (passive Heymann nephritis [PHN]), rats with toxic podocyte injury (puromycin aminonucleoside nephrosis [PAN]), rats developing a spontaneous glomerulosclerosis (Milan normotensive rats), and obese Zucker rats with hyperlipidemic and type II diabetic renal damage. Although PDGF-B is a known potent mesangial mitogen in vivo, a potential role of PDGF-C in mesangioproliferative glomerulonephritis in vivo is currently unknown. To address whether PDGF-C, which at least partially binds to the same receptors as PDGF-B, uses different pathways than PDGF-B in mediating mesangial cell proliferation in vivo, we also analyzed whether inhibition of PDGF-B (via PDGF-B specific aptamers) influenced the expression of PDGF-C in the anti-Thy 1.1 glomerulonephritis model.

Materials and Methods

Animal Models

All animal studies were approved by the local Institutional Review Board.

Normal Rats. Eight male Wistar rats (Charles River Wiga GmbH, Sulzfeld, Germany) weighing 140 to 180 g remained untreated and served as healthy control animals.

Mesangioproliferative Anti-Thy 1.1 Nephritis. Anti-Thy 1.1 nephritis was induced in 16 male Wistar rats (Charles River Wiga GmbH; weighing 160 to 180 g at the start of the experiment) by intravenous injection of 1 mg/kg monoclonal anti-Thy 1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK) as described (9). Animals remained untreated and were sacrificed at days 1, 4, 6, and 85 after induction of the disease (n = 4 at each time point).

Antagonism of PDGF-B after Induction of Anti-Thy 1.1 Glomerulonephritis. PDGF-C expression was additionally studied in ten rats that were treated with PDGF-B–specific or irrelevant (scrambled) control aptamers as described (9). Anti-Thy-1.1 nephritis was induced in ten male Wistar rats (Charles River Wiga GmbH) as described above. Rats were treated with aptamers from days 3 until sacrifice at day 6 after disease induction. Treatment consisted of twice-daily intravenous bolus injections of the substances dissolved in 400 μl of phosphate-buffered saline (PBS), pH 7.4. Five rats received 0.66 mg/d PDGF-B specific aptamer, and five rats received 0.66 mg/d scrambled aptamer.

Passive Heymann Nephritis. PHN was induced in four male Sprague-Dawley rats (Charles River Wiga GmbH; weighing 230 to 240 g) by intravenous injection of 0.8 ml of sheep anti-Fx1a antibody per rat (10). Animals remained untreated and were sacrificed at day 8 after induction of the disease.

Puromycin Aminonucleoside Nephrosis. PAN was induced in two male Sprague-Dawley rats (Charles River Wiga GmbH; weighing 210 to 230 g) by intravenous injection of 150 mg/kg puromycin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) dissolved in normal saline as described (11). Renal tissues were obtained after sacrifice on day 7.

Milan Normotensive Rats. Two Milan normotensive rats were obtained from the Hannover Medical School, Germany. Renal tissues were obtained after sacrifice at 40 wk of age (12).

Obese Zucker Rats. Three male obese (fa/fa) Zucker rats were obtained from Charles River Wiga. Renal tissues were obtained after sacrifice at 60 wk of age (13).

Tissues for morphologic evaluation and immunohistochemical analyses were fixed in methyl Carnoy solution, embedded in paraffin, and sectioned. The presence of morphologic features of the different renal diseases was examined in periodic acid-Schiff–stained sections. All tissue sections contained a minimum of 50 (usually >100) glomerular cross-sections.

Antibodies

Rabbit antisera directed against PDGF-C were generated as described previously in detail (7). Three different antisera preparations were used in the study: (1) anti-human full-length PDGF-CC, affinity-purified against the core domain of PDGF-CC; (2) anti-human PDGF-CC core domain, affinity-purified against the core domain of PDGF-CC; and (3) anti-mouse PDGF-CC peptide, affinity-purified against the core domain of PDGF-CC. Specificity of these antisera for the detection of PDGF-C has been demonstrated previously by Western blot analyses (7). Further analysis of the specificity of the antisera in immunohistochemical procedures was performed as part of the present study and is detailed below.

Rat renal collecting duct epithelial cells were detected with a rabbit polyclonal antibody directed against rat aquaporin-2 (14). Aquaporin-2 is a vasopressin-regulated water channel expressed exclusively in the renal collecting duct. The antibody was a kind gift of Dr. Mark Knepper, Renal Mechanisms Section, NHLBI, NIH, Bethesda, MD, USA. Additional primary antibodies were identical to those described previously (15,16) and included a murine monoclonal antibody (clone 1A4) to smooth muscle actin, a murine monoclonal antibody (clone PGF-007) to PDGF B-chain, a murine monoclonal IgG antibody (clone ED1) to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells, affinity-purified polyclonal goat anti-human/bovine type IV collagen IgG preabsorbed with rat erythrocytes, an affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody, and appropriate negative controls as described previously (15,16).

Immunohistochemical Analyses

Immunohistochemical analyses were performed following previously published protocols (17,18). Briefly, methyl Carnoy-fixed, paraffin-embedded tissues were sectioned at 4 μ. Sections were deparaffinized in xylene and rehydrated in graded ethanols. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. The sections were then incubated for 1 h with the primary antibody diluted in PBS containing 1% bovine serum albumin (Sigma). After washes in PBS, the sections were sequentially incubated with biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA), the ABC-Elite reagent (Vector), and finally 3,3′-diaminobenzidine (DAB, Sigma) with nickel chloride enhancement used as the chromogen. Sections were counterstained with methyl green, mounted, and coverslipped. Negative controls consisted of replacement of the primary antisera with nonimmune rabbit serum. In pilot experiments, we tested the sensitivity and specificity of our procedure. The antibodies were tested at final concentrations between 1 and 15 μg/ml. For the evaluation of the immunoperoxidase stains for α-smooth muscle actin, PDGF-B, and PDGF-C, each glomerular area was graded semiquantitatively, and the mean score per biopsy was calculated. Each score mainly reflects changes in the extent rather than intensity of staining and depends on the percentage of the glomerular tuft area showing focally enhanced positive staining: I, 0% to 25%; II, 25% to 50%; III, 50% to 75%; IV, >75%. We have recently described that data obtained using this scoring system are highly correlated with those obtained by computerized morphometry (19).
Cell Cultures/Mesangial Cell Proliferation Assay

The generation of primary rat mesangial cells has been previously described in detail (20). Cells were grown in RPMI 1640 (Sigma) supplemented with 15% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37°C in 5% CO2 and were passaged at subconfluency by harvesting with trypsin/ethylenediaminetetraacetic acid. The incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA was used as a measurement of mesangial cell proliferation. Mesangial cells (3 × 10^3 cells/well) were transferred to a 96-well microtiter plate and grown in RPMI 1640 containing 15% FCS until the cells were subconfluent. After incubation for 24 h in RPMI 1640 with 0.5% FCS, cells were stimulated for another 24 h with purified PDGF-CC protein (1 to 50 ng/ml [7]), PDGF-BB protein (1 to 50 ng/ml), PDGF-AA protein (1 to 50 ng/ml) (PDGF-AA and PDGF-BB were kindly provided by J. Hoppe, University of Würzburg, Germany). Cells were labeled with BrdU during the last 4 h of culture according to the manufacturers instructions (Cell proliferation enzyme-linked immunosorbent assay [ELISA], Roche Diagnostics GmbH, Mannheim, Germany). At the end of the incubation period, adherent mesangial cells were washed and denatured. Incorporated BrdU was detected by using a peroxidase-labeled anti-BrdU antibody and a peroxidase color substrate. Finally, the absorbance of the samples was measured in an ELISA reader at 370 nm. Cell proliferation experiments were independently performed four times with duplicate measurements.

Western Blot Analyses

PDGF-C expression was additionally analyzed in protein lysates from cultured primary rat mesangial cells from primary rat smooth muscle cells and from isolated glomerular fractions as described (21). Briefly, cells or isolated glomeruli were homogenized in Triton X-100 lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM MgCl2; 1 mM ethyleneglycoltetraacetic acid; 10% glycerol; 1% Triton X-100; 1 μg/ml aprotinin; 1 μg/ml leupeptin; 1 mM phenylmethyl sulfonyl chloride; 0.1 mM sodium orthovanadate) at 4°C. Incubation for 5 min preceded ultrasound treatment (3 × 10 s). The protein concentrations of the resulting solutions were determined using the BCA protein assay (Pierce, Rockford, IL). Forty micrograms of protein were electrophoresed under reducing conditions on a 10% sodium dodecyl sulfate gel and then blotted onto nitrocellulose membranes. The blots were blocked with 2% bovine serum albumin (Sigma) in TTBS (150 mM NaCl; 10 mM Tris, pH 8.0; 0.05% (vol/vol) Tween 20) for 1 h at room temperature and then incubated with the anti-human PDGF-CC core domain antibody diluted in TTBS overnight at 4°C. After several washes in TTBS, the blots were incubated with peroxidase-conjugated horse anti-rabbit IgG antibody (Vector) for 1 h. The blots were visualized with the enhanced chemiluminescence reagent (ECL; Amersham Pharmacia Biotech, Freiburg, Germany).

Statistical Analyses

Data are given as mean ± SD. Statistical significance, defined as P ≤ 0.05, was evaluated by using the t test.

Results

PDGF-C Expression in Normal Adult Rat Kidney

The immunohistochemical pattern of PDGF-C expression demonstrated a very consistent result in all eight analyzed animals (Table 1). The majority of glomeruli of normal rats were completely negative for PDGF-C (Figure 1A). Very few glomeruli of each case showed some weak granular PDGF-C immunohistochemical signal. However, this weak staining could not be clearly attributed to a specific glomerular cell type. Parietal epithelial cells were negative for PDGF-C. PDGF-C expression was regularly identified in the vascular compartment. Smooth muscle cells of arteries and arterioles constitutively expressed PDGF-C (Figure 1C). The immunohistochemical signal localized within the cytoplasm of smooth muscle cells and occasionally appeared to reflect a granular localization.

Table 1. PDGF-C protein expression in normal and diseased adult rat kidneys

<table>
<thead>
<tr>
<th>Glomeruli</th>
<th>Normal</th>
<th>Thy 1.1</th>
<th>PAN</th>
<th>PHN</th>
<th>Milan</th>
<th>fa/fa</th>
</tr>
</thead>
<tbody>
<tr>
<td>mesangial cells</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
<td>(+/−)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>endothelial cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>visceral epithelial cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>parietal epithelial cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>Vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>endothelial cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>smooth muscle cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>adventitial cells</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Tubulointerstitium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proximal tubules</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>distal tubules</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>loop of Henle</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>collecting ducts</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>interstitial cells</td>
<td>+</td>
<td>−</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

Normal, normal adult rat; Thy 1.1, mesangioproliferative anti-Thy 1.1 glomerulonephritis, day 6; PAN, puromycin aminonucleoside nephrosis; PHN, passive Heymann nephritis; Milan, Milan normotensive glomerulosclerosis; fa/fa, obese (fatty) Zucker rats. For details see Materials and Methods and Results sections.

−, no expression detectable; +/−, weak and/or variable expression; +, detectable expression.
deposition within the cells. PDGF-C expression remained undetectable in endothelial cells of all analyzed cases. Within the tubulointerstitial compartment, PDGF-C expression localized to a limited number of tubular structures (Figure 1E). Serial sections labeled with an antibody directed against aquaporin-2 clearly identified the PDGF-C–expressing tubular segments as collecting ducts (Figure 1F). Both cortical and outer medullary collecting ducts expressed PDGF-C. Proximal as well as distal tubular epithelial cells were negative for PDGF-C. Negative control tissue sections that were incubated with equal amounts of nonimmune rabbit serum did not demonstrate any staining signal in all analyzed normal and diseased cases.

Mesangial PDGF-C is Markedly Upregulated during Experimental Mesangioproliferative Glomerulonephritis

The anti-Thy 1.1 glomerulonephritis is a model of an antibody-induced, complement-mediated mesangiolysis, followed by a phase of mesangial cell proliferation with peak proliferation between 4 and 6 d after disease induction, and finally spontaneous restitution of normal glomerular morphology within 2 to 3 mo. Typical histologic features of this disease model were observed in the 16 animals analyzed in the present study. Glomerular PDGF-C expression was markedly altered in the course of the mesangioproliferative glomerulonephritis as compared with normal rats. By Western blot analyses, an upregulated glomerular PDGF-C expression was identified in the course of the glomerulonephritis (Figure 2). Although PDGF-C was undetectable in protein lysates from isolated glomeruli obtained from normal rats, an intense band corresponding with the proteolytically activated 33-kD PDGF-C protein could be identified in glomeruli obtained from rats at day 6 after anti-Thy 1.1 disease induction. This result was confirmed and further refined by our immunohistochemical studies (Table 1). Very early, at day 1 following disease induction, a weak granular immunohistochemical signal was observed in most glomeruli. The granular signal was detectable in a mesangial distribution pattern. In parallel to the peak mesangial cell activation/proliferation at days 4 and 6, there was a marked increase in mesangial PDGF-C expression (Figure 3). The immunohistochemical signal specifically localized to the mesangium, and glomerular endothelial cells and visceral epithelial cells remained negative for PDGF-C (Figure 3). In addition to the prominent mesangial expression, PDGF-C was detected in parietal epithelial cells of individual glomeruli at day 6 (data not shown). Circulating leukocytes within glomerular microaneurysms typically present at days 4 and 6 represented another frequent localization of glomerular PDGF-C. With the resolution of the glomerular injury at day 85, the majority of glomeruli became negative for PDGF-C. A limited number of glomeruli at day 85 continued to demon-

Figure 1. Platelet-derived growth factor–C (PDGF-C) protein expression in normal adult rat kidney. (A) In the normal rat kidney, PDGF-C is expressed in arteriolar smooth muscle cells and in epithelial cells of individual tubular segments (arrows). (B) Higher power magnification illustration of the same glomerulus as illustrated in panel A, showing PDGF-C localized to smooth muscle cells of afferent and efferent arteriole (arrows). Note the absent PDGF-C expression within the glomerulus. (C) Small cortical artery with smooth muscle cell PDGF-C positivity. (D) Serial section of panel C immunohistochemically stained for α-smooth muscle actin as marker of smooth muscle cells showing a virtually identical signal distributional pattern as seen for PDGF-C. (E) PDGF-C is expressed in a subset of tubular segments. (F) Serial section labeled for aquaporin-2 clearly identifies the tubular segments as collecting ducts. Magnifications: ×400 in A, E, and F; ×600 in B through D.

Figure 2. Western blotting for the expression of PDGF-C. (A) Cell lysates from primary rat smooth muscle cells (lane 1) and from primary rat mesangial cells (lane 2) were electrophoresed, blotted, and incubated with the anti-human PDGF-C core domain antiserum as detailed in the Materials and Methods section. A 33-kD band corresponding to the expected size of proteolytically cleaved, active PDGF-C is detected in lysates from both cell types. (B) Protein isolates from sieved glomeruli from normal rats (lane 1) and from rats at day 6 after induction of anti-Thy 1.1 glomerulonephritis induction (lane 2). Although glomeruli obtained from normal rats exhibit a very faint band only, glomerular PDGF-C expression is markedly upregulated at day 6 after glomerulonephritis induction.
strate a segmental PDGF-C staining pattern that mirrored areas of focal mesangial cell proliferates persisting after the initial injury. PDGF-C expression remained unchanged in the tubulointerstitial and the vascular compartments as compared with normal rats.

The use of the three different antisera directed against full-length human PDGF-C, core domain human PDGF-C, and mouse PDGF-C peptide resulted in virtually identical tissue localization of PDGF-C in rat renal tissues. As illustrated in Figure 3, serial sections obtained from a rat at 4 d after induction of a anti-Thy 1.1 nephritis, PDGF-C localized to activated mesangial cells was equally detected by the three different antisera preparations. This result further indicates that, due to the high degree of conservation of the amino acid sequences between mammalian species, both anti-human as well as anti-mouse PDGF-C antibody preparations are cross-reactive with rat PDGF-C.

**PDGF-C Acts as a Growth Factor for Mesangial Cells In Vitro**

Cultured primary rat mesangial cells were growth arrested and stimulated with PDGF-AA, PDGF-BB, and PDGF-CC. Cell proliferation assays revealed that treatment with purified PDGF-CC protein led to a significant induction of mesangial cell proliferation at PDGF-CC concentrations 10 ng/ml (Figure 4A). Further increase in PDGF-CC concentrations resulted in a dose-dependent increase in mesangial cell proliferation. As expected from numerous previous experiments, both known PDGF-isoforms, PDGF-BB and to a lesser degree PDGF-AA,

![Figure 3](image_url)

**Figure 3.** Mesangial PDGF-C is markedly upregulated during experimental mesangio proliferative glomerulonephritis. Serial sections of a kidney obtained from a rat 4 days after induction of a mesangio proliferative anti-Thy 1.1 glomerulonephritis. High power magnification illustrations demonstrate expression of PDGF-C within the activated glomerular mesangium. (A) Immunohistochemical analyses performed with anti-human full-length PDGF-C results in a virtually identical staining pattern compared with immunohistochemical analyses performed with anti-human core domain PDGF-C antiserum (B), or anti-mouse PDGF-C antiserum (C). The glomerular expression of PDGF-C strongly correlates with the expression of smooth muscle α-actin, a marker of rat mesangial cell activation in vivo (D). Staining for α-actin identifies activated glomerular mesangial cells and arteriolar smooth muscle cells (upper right) and periglomerular myofibroblasts. A more pronounced upregulation of PDGF-C in the mesangium is present on day 6 after disease induction (E). Magnifications, × 600.

![Figure 4](image_url)

**Figure 4.** PDGF-C acts as growth factor for mesangial cells in vitro. (A) BrdU incorporation into growth arrested mesangial cells stimulated with control medium, PDGF-AA (1 to 50 ng/ml), PDGF-BB (1 to 50 ng/ml), or PDGF-CC (1 to 50 ng/ml). Data are mean ± SD of four independent experiments. *P < 0.05 versus control. (B) BrdU incorporation into growth arrested mesangial cells stimulated with control medium, PDGF-BB (50 ng/ml), PDGF-BB (50 ng/ml) with PDGF-B aptamer (1 μg/ml), PDGF-BB with control scrambled aptamer (1 μg/ml), PDGF-C aptamer (1 μg/ml), PDGF-CC (50 ng/ml), PDGF-C aptamer (1 μg/ml), or PDGF-CC (50 ng/ml) with control scrambled aptamer (1 μg/ml). Data are mean ± SD of four independent experiments. **P < 0.05 versus no aptamer.
were capable of inducing dose-dependent mesangial proliferation in similar concentration (Figure 4A).

**PDGF-B Antagonism Does Not Alter Mesangial PDGF-C Expression**

Treatment of nephritic rats with PDGF-B specific aptamer antagonists resulted in a decrease in mesangial cell proliferation and glomerular matrix accumulation at day 6 in the course of the anti-Thy 1.1 glomerulonephritis as compared with rats treated with control irrelevant aptamers. The mesangial overexpression of PDGF-C was not altered by the PDGF-B antagonism. Immunostaining scores of the PDGF-C staining demonstrated no significant differences between PDGF-B aptamer-treated nephritic rats (1.6 ± 0.4) and control aptamer-treated nephritic rats (1.6 ± 0.4). The glomerular overexpression of PDGF-B chain was significantly reduced in PDGF-B aptamer-treated nephritic rats (1.8 ± 0.9) compared with control aptamer-treated nephritic rats (2.9 ± 0.6) in parallel with a significant reduction of mesangial cell activation (α-smooth muscle-actin immunostaining scores in PDGF-B aptamer-treated nephritic rats 1.7 ± 0.2 compared with control aptamer-treated nephritic rats 2.3 ± 0.5). Additional in vitro experiments confirmed that the PDGF-B aptamer, although specifically antagonizing the mitogenic activity of PDGF-BB, did not affect the mitogenic effect of PDGF-CC on cultured rat mesangial cells (Figure 4B).

**PDGF-C Expression in Experimental Glomerular Diseases Primarily Associated with Podocyte Injuries**

We next analyzed the expression of PDGF-C in rat experimental glomerular disease models that were primarily associated with injury to visceral epithelial cells (Table 1).

Animals with toxic podocytic injury (PAN) developed massive proteinuria ranging from 210 to 300 mg/d. Glomeruli remained largely negative for PDGF-C (Figure 5A). Several glomeruli demonstrated a weak granular PDGF-C expression following a mesangial distribution. Visceral epithelial cells, e.g., the primary targets of the experimental disease, remained negative for PDGF-C (Figure 5A). Both, arterial smooth muscle cell and collecting duct PDGF-C expression remained unchanged as compared with normal rats.

The development of significant proteinuria (80 to 170 mg/d) was also the functional hallmark of the immune-mediated podocyte injury (PHN). However, despite the presence of severe glomerular injury resembling human membranous nephropathy, the majority of glomeruli did not show detectable PDGF-C expression (Figure 5B). A weak granular mesangial PDGF-C expression was identified in a very limited number of glomeruli. PDGF-C expression was additionally identified in a few individual circulating leukocytes within glomerular capillary lumina. Parietal epithelial cells of some glomeruli were also positive for PDGF-C (Figure 5B). The expression of PDGF-C in collecting ducts and arterial smooth muscle cells remained normal.

Milan normotensive rats spontaneously develop focal segmental glomerulosclerosis. Visceral epithelial cells had been identified as the primary target of injury in this model (12). The majority of the glomeruli that appeared histologically normal contained no detectable PDGF-C. However, at sites of focal glomerular injury, PDGF-C was localized to visceral epithelial cells (Figure 5C). Glomerular endothelial cells and mesangial cells had no detectable PDGF-C. Another frequent finding was the PDGF-C expression of parietal epithelial cells. PDGF-C expression in vascular smooth muscle cells and in collecting ducts was unchanged.

Obese (fa/fa) Zucker rats develop hyperlipidemia and type IIb diabetes with progressive glomerulosclerosis and tubulointerstitial damage. The four 60-wk-old rats demonstrated significant functional alterations (increased urinary protein excretion, increased plasma creatinine) and significant histologic alterations (focal and segmental glomerulosclerosis, tubulointerstitial fibrosis, tubular atrophy) (13). The glomeruli demonstrated a wide range from histologically minimal lesions to segmental sclerosis lesions and finally globally sclerotic glomeruli. Despite the presence of severe glomerular pathology, glomerular PDGF-C expression remained negative in all cases (Figure 5D).

**Increase in PDGF-C Expression at Sites of Fibrosing Tubulointerstitial Injury**

Most analyzed models of glomerular disease did not develop significant tubulointerstitial pathology because of either the resolution of the injury (anti-Thy 1.1 nephritis) or the early sacrifice after disease induction (day 8 in PAN and PHN). However, 60-wk-old obese diabetic Zucker rats exhibited severe tubulointerstitial disease, including tubular atrophy, tubu-

![Figure 5. PDGF-C expression in experimental glomerular diseases primarily associated with podocyte injuries. (A) Representative glomerulus of a puromycin nephrosis rat immunohistochemically labeled for PDGF-C. Arteriolar smooth muscle cells express PDGF-C but glomerular cells remain negative for PDGF-C despite severe podocytic injury. (B) Absent glomerular PDGF-C expression in a glomerulus after passive Heymann nephritis disease induction. PDGF-C expression can be detected in parietal epithelial cells (arrows). (C) Representative glomerulus of a Milan rat demonstrating weak PDGF-C expression in individual visceral epithelial cells (arrows). (D) Absent PDGF-C expression in glomeruli of obese (fa/fa) diabetic rats. Magnification, × 600.](image-url)
lar microcystic dilation, and tubulointerstitial accumulation of leukocytes and myofibroblasts. At these sites of injury, tubulointerstitial PDGF-C expression was markedly upregulated in all cases analyzed (Figure 6, A and C). PDGF-C was restricted to interstitial cells. The PDGF-C expression pattern of collecting duct epithelial cells remained unchanged compared with normal rats. We were unable to clearly identify the phenotypes of these interstitial PDGF-C expressing cells. However, serial immunohistochemical staining detected ED-1^+ macrophages in a similar distribution as PDGF-C–expressing cells (Figure 6).

**Discussion**

Beyond the demonstration of abundant PDGF-C transcripts in human kidney (7), no information is presently available on its intrarenal localization or pathogenetic role. In normal rat kidney, we identified arterial and arteriolar smooth muscle cells and collecting ducts as sites of constitutive PDGF-C expression. The former is consistent with a recent study by Uutela et al. (22) that identified PDGF-C protein by immunohistochemistry in smooth muscle cells in sections from the suprarenal artery. In addition, PDGF-C stimulated proliferation of human coronary artery and aortic smooth muscle cells in vitro, suggesting that PDGF-C, like the known PDGF-A and PDGF-B isoforms, can participate in vascular development and pathology (22). The detection of constitutive PDGF-C expression in collecting duct epithelial cells is novel. However, the functional role of collecting duct PDGF-C expression remains unclear, in particular, given that the animals analyzed in the present study did not show any alterations of the expression pattern in the models analyzed.

In renal disease, one of the notable findings was a marked and apparent de novo upregulation of PDGF-C during the proliferative response phase of anti-Thy 1.1–induced mesangio proliferative glomerulonephritis. The upregulation appeared to selectively label activated mesangial cells and as such was reminiscent of findings with PDGF-A and PDGF-B in glomerular disease (5,6). Cultured mesangial cells express both PDGF receptors, and both ligands, PDGF-A and PDGF-B, can induce proliferation of this cell type, although PDGF-B is much more potent (5,6). A central role particularly of the PDGF-B-chain in mediating mesangial cell proliferation and matrix overproduction in vitro has been documented in several studies (5,6). Here we identify PDGF-C as the third family member that is capable of inducing mesangial cell proliferation in vitro with a mitogenic activity that is more potent than PDGF-AA yet lower than that of PDGF-BB. Additionally, our study identifies mesangial cells not only as a target but also as a source of PDGF-C production in vitro and in vivo, indicating the existence of an autocrine stimulatory pathway similar to that previously described for PDGF-B (5). To gain further insight into the regulation of PDGF-C in vivo and in particular a potential interplay with PDGF-B, we also assessed the mesangial PDGF-C expression during anti-Thy 1.1 nephritis after specific antagonism of PDGF-B. Although selective inhibition of PDGF-B had a significant effect on mesangial proliferation and matrix accumulation, the expression of mesangial PDGF-C was not altered by PDGF-B inhibition, indicating the existence of different pathways in the regulation of PDGF-B and PDGF-C.

Apart from glomerular mesangial cells, visceral and parietal epithelial cells have been identified as additional glomerular sources of apparent de novo PDGF-C expression in animal models with predominant injury to these cell types. These findings are again reminiscent of findings for PDGF-B, which can be expressed by visceral and parietal glomerular epithelial cells after injury (23,24). In the case of both PDGF-B and -C, the absence of functional in vitro and particularly in vivo studies presently precludes speculations on the role of PDGF-B and -C in these cell types. However, as in the case of PDGF-B (25), it is noteworthy that there was no clear correlation between the degree of glomerular sclerosis and the extent of glomerular epithelial PDGF-C expression.

A significant increase in individual PDGF-C–expressing cells was seen at sites of fibrosing interstitial injury that was most prominent in the hypercholesterolemic, diabetic rats. Although the exact cellular origin of PDGF-C expression remains to be determined, this study identified monocytes/macrophages as potential sources of PDGF-C in this scenario. However, the additional expression of PDGF-C in a small subset of fibroblasts or myofibroblasts cannot be formally excluded. Li et al. (7) identified PDGF-C as a potent mitogen for cultured fibroblast cells, and Gilbertson et al. (8) have been able to demonstrate PDGF-C–induced proliferation of several mesenchymal cell types, including human adventitial fibroblasts in vitro. Li et al. (7) have furthermore linked PDGF-C expression with fibrosis. Transgenic expression of PDGF-C in the mouse heart, using the promoter for the α-myosin heavy chain, induced strong proliferation of cardiac fibroblasts and subsequent interstitial expansion with features of interstitial fibrosis (7). In the renal interstitium, the PDGF-α-receptor is constitutively expressed (7) and PDGF β-receptor is upregulated in progressive renal failure (19). Therefore, the de novo occurrence of PDGF-C–producing cells in damaged renal tubulointerstitium may contribute to the pathogenesis of renal tubulointerstitial fibrosis.

In conclusion, this study links pathologic overexpression of the new growth factor PDGF-C to the induction of mesangial cell proliferation in vivo and in vitro as well as to tubulointer-
stitial inflammation and fibrosis. Compounds that specifically inhibit PDGF-C in vivo will help to further define the role of PDGF-C in the concert of actions of the members of the PDGF family of cytokines.

Acknowledgments

The technical help of Kerstin Diekmann, Gabi Dietzel, Gerti Mi-
nartz, and Andrea Cosler is gratefully acknowledged. This work was supported in part by grant SFB 542, project C7 from the Deutsche Forschungsgemeinschaft (DFG), by a grant from the Swedish Research Council (grant K2001–03P–12070–05B) and the Novo Nor-
disk Foundation. FE is a recipient of a stipend of the German Kidney Foundation (Deutsche Nierenstiftung). The authors are grateful for Judy Ruckman, Gilead Sciences Inc., Boulder, CO, USA for providing the aptamer reagents.

References

10. Salant DJ, Darby C, Couser WG: Experimental membranous glomerulonephritis in rats. Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole ani-
J, Kuhn KW, Koch KM, Brunkhorst R: Age-related glomeru-
14. DiGiovanni SR, Nielsen S, Christensen EI, Knepper MA: Regu-
lation of collecting duct water channel expression by vasopres-
15. Burg M, Ostendorf T, Mooney A, Koch KM, Floege J: Treat-
17. Floege J, Hudkins KL, Davis CL, Schwartz SM, Alpers CE: Ex-
pression of PDGF alpha-receptor in renal arteriosclerosis and re-
HJ, Floege J: Differential activation of mitogen-activated protein kinases in experimental mesangio proliferative glomerulonephri-
22. Uutela M, Lauren J, Bergsten E, Li X, Horelli-Kuitunen N, Eri-
25. Floege J, Burns MW, Alpers CE, Yoshimura A, Pritzl P, Gordon K, Seifert RA, Bowen-Pope DF, Couser WG, Johnson RJ: Glo-
merular cell proliferation and PDGF expression precede glomeru-