A Fully Human Monoclonal Antibody (CR002) Identifies PDGF-D as a Novel Mediator of Mesangioproliferative Glomerulonephritis

TAMMO OSTENDORF,* CLAUDIA R.C. VAN ROEYEN,* JEFFREY D. PETERSON,† UTA KUNTER,* FRANK EITNER,* AVIN J. HAMAD,* GERLINDE CHAN,† XIAO-CHI JIA,‡ JENNIFER MACALUSO,† GADI GAZIT-BORNSTEIN,‡ BRUCE A. KEYT,‡ HENRI S. LICHENSTEIN,† WILLIAM J. LAROCHELLE,† and JÜRGEN FLOEGE*  
*Division Nephrology, University of Aachen, Germany; †CuraGen Corporation, Branford, Connecticut; and ‡Abgenix, Inc., Fremont, California

Abstract. PDGF-B is of central importance in mesangioproliferative diseases. PDGF-D, a new PDGF isoform, like PDGF-B, signals through the PDGF ββ-receptor. The present study first determined that PDGF-D is mitogenic for rat mesangial cells and is not inhibited by a PDGF-B antagonist. Low levels of PDGF-D mRNA were detected in normal rat glomeruli. After induction of mesangioproliferative nephritis in rats by anti-Thy 1.1 mAb, glomerular PDGF-D mRNA and protein expression increased significantly from days 4 to 9 in comparison with nonnephritic rats. Peak expression of PDGF-D mRNA occurred 2 d later than peak PDGF-B mRNA expression. In addition, PDGF-D serum levels increased significantly in the nephritic animals on day 7. For investigating the functional role of PDGF-D, neutralizing fully human mAb were generated using the XenoMouse technology. Rats with anti-Thy 1.1–induced nephritis were treated on days 3 and 5 with different amounts of a fully human PDGF-DD–specific neutralizing mAb (CR002), equal amounts of irrelevant control mAb, or PBS by intraperitoneal injection. Specific antagonism of PDGF-D led to a dose-dependent (up to 67%) reduction of glomerular cell proliferation. As judged by double immunostaining for 5-bromo-2'-deoxyuridine and α-smooth muscle actin, glomerular mesangial cell proliferation was reduced by up to 57%. Reduction of glomerular cell proliferation in the rats that received CR002 was not associated with reduced glomerular expression of PDGF-B mRNA. PDGF-D antagonism also led to reduced glomerular infiltration of monocytes/macrophages (day 5) and reduced accumulation of fibronectin (day 8). In contrast, no effect was noted in normal rats that received an injection of CR002. These data show that PDGF-D is overexpressed in mesangioproliferative states and can act as an auto-, para-, or even endocrine glomerular cell mitogen, indicating that antagonism of PDGF-D may represent a novel therapeutic approach to mesangioproliferative glomerulonephritides.

For two decades, the PDGF system consisted of two PDGF chains, PDGF-A and -B, that are secreted as homo- or heterodimers and bind to dimeric PDGF receptors composed of α- and/or β-chains. Whereas PDGF-A binds to the α-chain only, PDGF-B is a ligand for all receptor types (1). Recently, two novel PDGF isoforms, designated PDGF-C and -D, that are released as homodimers, PDGF-CC and -DD, were described (2–4). The core chain of PDGF-CC seems to be largely a ligand for the PDGF αα-receptor, whereas PDGF-DD binds predominantly to the PDGF ββ-receptor (2–4). In both cases, some binding has also been described to the αβ-receptor (2,4,5). All four PDGF isoforms, as well as both receptor chains, are expressed in the kidney, albeit in distinct spatial arrangements (1,6,7).

Many progressive renal diseases, including diabetic nephropathy, as well as the most frequent types of glomerulonephritides, such as IgA nephropathy, are characterized by glomerular mesangial cell proliferation and/or matrix accumulation (8). Ample evidence is now available to link the PDGF system, in particular PDGF-B-chain, to both of these processes given that (1) mesangial cells produce PDGF-B in vitro and various growth factors induce mesangial proliferation via induction of auto- or paracrine PDGF-B-chain expression (9–12); (2) PDGF-B-chain and its receptor are overexpressed in many glomerular diseases (13–17); (3) infusion of PDGF-BB or glomerular transfection with a PDGF-B-chain cDNA can induce selective mesangial cell proliferation and matrix accumulation in vivo (18,19); (4) PDGF-B-chain or β-receptor knockout mice fail to develop a mesangium (20,21); and (5) specific inhibition of PDGF-B using antibodies, aptamers, soluble PDGF receptors, or PDGF β-receptor tyrosine kinase blockers not only results in a diminution of mesangioprol-
liferative changes (reviewed in (1) and (22–24)) but, more important, was also able to prevent long-term renal scarring (25). Apart from its role in glomerular pathology, PDGF-B-chain may also contribute to renal interstitial fibrosis, i.e., the common final pathway of almost all progressive renal diseases. Thus, we have reported that both PDGF-β-receptor subunit and PDGF-B-chain are overexpressed in renal interstitial fibrosis (26), and Tang et al. (27) demonstrated that infusion of large doses of PDGF-BB alone is able to induce interstitial fibrotic changes in normal rat kidney.

Given that PDGF-D, like the PDGF-B-chain, can bind to the ββ- and αβ-receptor, their biologic activities in the kidney may be similar, albeit not identical, in view of the differential binding to the PDGF αα-receptor. PDGF-DD is secreted as a disulphide-linked homodimer, which is activated upon limited proteolysis with dissociation of its CUB-domain to become a disulphide-linked homodimer, which is activated upon limited proteolysis with dissociation of its CUB-domain to become a specific agonistic ligand for PDGF ββ- and αβ-receptor (2,4).

In developing and adult normal kidney, PDGF-D is expressed in visceral glomerular epithelial cells and some vascular smooth muscle cells (6). In the developing mouse kidney, only cells of the branching ureter exhibited PDGF-D immunoreactivity (4). No other information on PDGF-D and the kidney is currently available. In the present study, we investigated whether PDGF-D mediated glomerular mesangial cell proliferation and matrix accumulation in vitro and in vivo.

Materials and Methods

Reagents

Recombinant human and murine PDGF-DD lacking the CUB-domain, i.e. biologically active PDGF-DD p35, was produced as described (2). Human PDGF-CC was produced by the same protocol. PDGF-AA and PDGF-BB were purchased from R & D Systems (Minneapolis, MN). Immunohistochemical analysis was performed with a PDGF-DD-specific rabbit polyclonal antibody as described previously (15).

Fully human PDGF-DD mAb were generated as described previously (28) with the following modifications. Briefly, the human IgG2-bearing XenoMouse strain (8 to 10 wk old) was immunized twice weekly by footpad injection with 10 μg of V5-tagged soluble PDGF-DD (2) in complete Freund’s adjuvant (28). Hybridomas were generated using electrotoc fusion. Fully human isotype matched mAb PK16.3 was used as the negative control.

Characterization of the Neutralizing Anti-PDGF-D mAb CR002.6.4

PDGF solid-phase ELISA was performed by coating Corning 96-well flat-bottom high-protein binding polystyrene microtiter plates with 500 ng/ml human PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD or murine PDGF-DD overnight. Plates were blocked with Assay Dileu (Pharmingen, San Diego, CA) for 1 h. PDGF-DD mAb CR002.6.4 or control mAb PK16.3 was then added at the indicated concentration for 2 h. Primary mAb binding was detected using anti-human horseradish peroxidase–conjugated secondary antibody with TMB Reagent (Pharmingen). Microtiter plates were read at 450 nm with a Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA).

For Western blot analysis, human PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD (250 ng) or glomerular protein lysates (20 μg) isolated from rats with anti-Thy 1.1 nephritis day 8 after disease induction were subjected to 16% SDS-PAGE (human PDGF) or 4 to 20% SDS-PAGE (rat glomerular protein lysates) under nonreducing conditions. Proteins were transferred to Hybond-P membranes (Amersham), and filters were probed with mAb CR002.6.4 or control mAb PK16.3 (0.85 μg/ml) for 12 h. After washing, filters were incubated with anti-human horseradish peroxidase–conjugated secondary antibody. Bands were visualized by enhanced chemiluminescence (Amersham).

The NIH 3T3 and normal human lung fibroblast neutralization assay was performed as described previously (2) with the following modifications. Briefly, cells were serum-starved 24 or 18 h, respectively, and mAb were added at the indicated concentration. Human or murine PDGF-DD was then added at 100 ng/ml. After 18 h, 5-bromo-2′-deoxyuridine (BrdU) was added for 5 h and incorporation-assayed according to the manufacturer’s specifications (Roche).

Aptamer-Based Antagonist against PDGF

The synthesis and characterization of the PDGF-B aptamer (NX1975) have been described in detail (29). Modifications of the original DNA aptamer involved substitutions of certain nucleotides with 2-fluoropyrimidines and 2′-O-methylpurines to improve nuclease resistance as well as coupling of the aptamer to 40-kD polyethylene glycol to prolong its plasma residence time in vivo (22).

Mesangial Cell Culture Experiments

Rat mesangial cells were established in culture, characterized, and maintained as described previously (30). For examining the proliferative effect of PDGF-DD, rat mesangial cells were seeded in 96-well plates (Nalge Nunc, Naperville, IL), grown to subconfluence, and growth-arrested for 48 h in RPMI 1640 with 1% BSA. After 48 h, human PDGF-DD (10 to 200 ng/ml) and human PDGF-BB (10 and 50 ng/ml) together with PDGF-B-chain aptamer (100 ng/ml) or sequence-scrambled aptamer (100 ng/ml) were added and the cells were incubated for 24 h. DNA synthesis was determined by a BrdU incorporation assay according to the manufacturer’s instructions (Roche).

Glomerular RNA Extraction and Analyses

Total RNA was extracted from isolated rat glomeruli and adrenal gland with the guanidinium isothiocyanate/phenol/chloroform method using standard procedures (31). The RNA content and sample purity were determined by UV spectrophotometry at 260 and 280 nm. The cDNA syntheses were performed in a 30-μl reaction mix including 1 μg of total RNA, 1 μl of random primer (6 nt, 250 ng/μl; Roche), 6 μl of M-MLV reverse transcriptase buffer (Invitrogen, Carlsbad, CA), 1.5 μl of dNTP-mix (10 mM each, Amersham Pharmacia Biotech), 0.7 μl of RNase-inhibitor (40 U/μl, Promega), 1 μl of M-MLV reverse transcriptase (200 U/μl; Invitrogen), and DEPC-treated H2O. The mix was incubated for 10 min at 25°C followed by 1 h at 42°C.

Real-time quantitative PCR was carried out using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). In each reaction, 0.75 μl of cDNA and 12.5 μl of PCR Master Mix (Platinum Quantitative PCR SuperMix-UDG with ROX Reference Dye; Invitrogen) were used in a total of 25-μl volume. The PCR conditions were 50°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Taqman primers and probes were designed from sequences in the GenBank database using the Primer Express software (Applied Biosystems). The sequences of primers and probes used in this study are listed in Table 1.

Rat Model of Mesangio proliferative Glomerulonephritis

All animal experiments were approved by the local review boards. Anti-Thy 1.1 mesangial proliferative glomerulonephritis was induced...
in male Wistar rats (Charles River) weighing 180 g by injection of 1 mg/kg anti-Thy 1.1 mAb (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, England).

For studying the kinetics of PDGF-D expression during anti-Thy 1.1 nephritis, 45 rats received anti-Thy 1.1 mAb and were killed at time points 4 h and days 1, 2, 4, 7, 9, 14, 21, and 28 after mAb injection (n = 5 each). After the animals were killed, renal tissue and isolated glomeruli were obtained. Glomerular isolation was performed by differential sieving (32). Glomerular isolates were checked microscopically and exhibited a purity of >95%. In addition, adrenal tissue was obtained.

For studying the effects of PDGF-DD antagonism in vivo, rats were treated with the anti–PDGF-D mAb CR002.6.4, control mAb PK16.3, or PBS on days 3 and 5 after disease induction. Treatment consisted of intraperitoneal injections of the mAb dissolved in 800 µl of 20 mM Tris-HCl/100 mM NaCl (pH 7.4). mAb timing was chosen to treat rats from approximately 1 d after onset to the peak of mesangial cell proliferation, which in the OX-7–induced anti-Thy 1.1 nephritis model occurs between days 5 and 8 after disease induction. We investigated the in vivo effects of three different doses of the anti–PDGF-D mAb. The average dose of 10 mg (day 3) plus 4 mg (day 5) anti–PDGF-D mAb CR002.6.4/kg body wt was chosen on the basis of calculations that this would result in serum levels of >50 µg/mL. For verifying that relevant levels of CR002.6.4 or irrelevant control IgG2 PK16.3 were achieved, human IgG2 serum levels were measured in treatment groups 1 to 4 (see below) on days 5 and 8. Animals with levels <30 µg/mL on day 5 were excluded from the analyses. Altogether, seven groups of rats with sufficient human serum IgG2 in the mAb-treated groups were studied:

1. Seven nephritic rats that received 5 mg/kg body wt CR002.6.4 on day 3 and 2 mg/kg on day 5
2. Seven nephritic rats that received 10 mg/kg body wt CR002.6.4 on day 3 and 4 mg/kg on day 5
3. Eight nephritic rats that received 20 mg/kg body wt CR002.6.4 on day 3 and 8 mg/kg on day 5
4. Eight nephritic rats that received 40 mg/kg body wt irrelevant control PK16.3 on day 3 and 8 mg/kg on day 5
5. Nine nephritic rats that received equivalent injections of PBS alone
6. Five nonnephritic, normal rats that received 10 mg/kg body wt of CR002.6.4 on day 3 and 4 mg/kg on day 5
7. Five nonnephritic, normal rats that received 10 mg/kg body wt irrelevant control PK16.3 on day 3 and 4 mg/kg on day 5

In four randomly selected rats each from groups 1 to 5, renal biopsies for histologic evaluation were obtained on day 5 by intravital biopsy as described (22). In all rats, post mortem biopsy was obtained on day 8 after disease induction. The remaining cortical tissue of two or three rats from each group was then pooled and used to isolate glomeruli (see above). Urine collections were performed on day 7 after disease induction. BrdU (100 mg/kg body wt; Sigma, St. Louis, MO) was injected intraperitoneally 4 h before killing on day 8.

### Renal Morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy’s solution and embedded in paraffin. Four-microgram sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. In the periodic acid-Schiff–stained sections, the number of mitoses in >30 cross-sections (range, 30 to 100) of consecutive cortical glomeruli containing >20 discrete capillary segments each was evaluated by one of the authors, who was unaware of the origin of the slides. Mesangiolysis was graded as described (33) on a semiquantitative scale: 0 = no mesangiolysis, 1 = segmental mesangiolysis, 2 = global mesangiolysis, 3 = microaneurysm.

### Immunoperoxidase Staining

Four-microgram sections of methyl Carnoy’s fixed biopsy tissue were processed by an indirect immunoperoxidase technique (34). Primary antibodies were identical to those described previously (33,35) and included a murine mAb (clone 1A4) to α-smooth muscle actin; a murine mAb (clone PGE-007) to PDGF-B-chain; a murine mAb (clone ED1) to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells; a polyclonal goat antibody to human type I collagen (Southern Biotechnology Associates, Birmingham, AL); an affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA); plus appropriate negative controls as described previously (33,35). PDGF-DD was detected by a polyclonal rabbit antibody to human PDGF-DD. Serum was purified by Protein A Sepharose chromatography. PDGF-CC cross-reactivity was eliminated by absorption to a PDGF-CC affinity column. The resulting Ig flowthrough was concentrated and did not react with PDGF-AA, -BB, or -CC by ELISA or Western blot analysis.

For obtaining mean numbers of infiltrating leukocytes in glomeruli, >50 consecutive cross-sections of glomeruli were evaluated and mean values per kidney were calculated. For the evaluation of the immunoperoxidase stains for type I collagen, fibronectin, and α-smooth muscle actin, each glomerular area was graded semiquantitatively and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than the intensity of staining and depends on the percentage of the glomerular tuft area showing focally enhanced positive staining: 1 = 0 to 25%, 2 = 25 to 50%, 3 = 50 to 75%, 4 = >75% (22). Evaluation of all slides was performed by an observer, who was unaware of the origin of the slides.

### Immunohistochemical Double Staining

Double immunostaining to identify the type of proliferating cells was performed as reported previously (26) by first staining sections for proliferating cells with a murine mAb (clone BU-1) against

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Taqman Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat GAPDH</td>
<td>5′-AACAGATGGTGAGTTGCGAGTGTG-3′</td>
<td>5′-AGAAAGGCCAGCCTGTTAACCC-3′</td>
<td>5′-CGGATTGCGCGTATCGGACG-3′</td>
</tr>
<tr>
<td>Rat PDGF-A</td>
<td>5′-TTCTTGGTACGGCTGCCCTACAT-3′</td>
<td>5′-TTGACCGTGTCCTTACGAGCCG-3′</td>
<td>5′-CAGTGCACGGCTTTCCACACCA-3′</td>
</tr>
<tr>
<td>Rat PDGF-B</td>
<td>5′-GCAAGACGCCTAGACACGGTG-3′</td>
<td>5′-GAAATGGCAATTGCTGGACCA-3′</td>
<td>5′-TCCAGATCTCAGGGACACCTCTAGCG-3′</td>
</tr>
<tr>
<td>Rat PDGF-C</td>
<td>5′-CCAGAACTCCTCTCTACGCGTG-3′</td>
<td>5′-GACACCTCTGCTACGCGTG-3′</td>
<td>5′-CGGACAGACAGACGGTAAGGCA-3′</td>
</tr>
<tr>
<td>Rat PDGF-D</td>
<td>5′-ATCGGAGACACTTGTGCGACT-3′</td>
<td>5′-GTGCGCCGTACCCGGAATGTT-3′</td>
<td>5′-TTGCCGAATGCGAACCTCACAGG-3′</td>
</tr>
</tbody>
</table>
BrdU-containing nuclease in Tris-buffered saline (Amersham) using an indirect immunoperoxidase procedure. Sections were then incubated with the IgG1 mAb 1A4 raised against α-smooth muscle actin. Cells were identified as proliferating mesangial cells when they showed positive nuclear staining for BrdU and when the nucleus was completely surrounded by cytoplasm positive for α-smooth muscle actin. Negative controls included omission of either of the primary antibodies, in which case no double staining was noted.

**Serum Measurements of PDGF-DD and Anti-CR002.6.4 mAb**

Circulating levels of rat PDGF-DD were measured using a PDGF-DD–specific sandwich ELISA as described previously with modifications to allow accurate quantification of rodent forms of PDGF-DD (36). The two fully human mAb (CR002.1.6 and CR002.1.17) used in the sandwich ELISA recognized different epitopes on the PDGF-DD molecule (data not shown). The ELISA was performed as follows: 50 μl of capture mAb (CR002.1.6) in coating buffer (0.1 M NaHCO₃, pH 9.6) at a concentration of 2 μg/ml was coated on ELISA plates (Fisher). After incubation at 4°C overnight, the plates were treated with 200 μl of blocking buffer (0.5% BSA, 0.1% Tween 20, 0.01% Thimerosal in PBS) for 1 h at 25°C. The plates were washed (3×) with washing buffer (WB; 0.05% Tween 20 in PBS) and were incubated with serum samples overnight at 4°C, washed with WB, and then incubated with 100 μl/well biotinylated detection mAb CR002.1.17 for 1 h at 25°C. After washing, the plates were incubated with horseradish peroxidase–streptavidin for 15 min, washed as before, and then treated with 100 μl/well o-phenylenediamine in H₂O₂ (Sigma developing solution) for color generation. The reaction was stopped with 50 μl/well H₂SO₄ (2 M) and analyzed using an ELISA plate reader at 492 nm. Concentration of anti–PDGF-D mAb in serum samples was calculated by comparison with dilutions of purified CR002.6.4 mAb using a four-parameter curve-fitting program.

**Miscellaneous Measurements**

Urinary albumin levels were determined with an ELISA kit specific for rat albumin (Nephrat, Exocell). Urinary creatinine was determined by the method of two-point kinetics with a Vitros 250 analyzer (Orthoclinical Diagnostics). All measurements were performed in duplicate. BP measurements were performed by the tail-cuff method, using a programmed sphygmomanometer, BP-98A (Softron) (37). Measurements of total hemolytic activity of the classic complement pathway (CH₅₀) and hemolytic activity of the alternative complement pathway (AP₅₀) were performed in sera of rats using appropriate assays (Total Hemolytic Complement and Alternative Pathway Hemolytic Complement kits; The Binding Site, Birmingham, England) according to the manufacturer’s instruction. All measurements were performed in duplicate.

Figure 1. PDGF-DD acts as a growth factor for mesangial cells in vitro. 5-Bromo-2′-deoxyuridine (BrdU) incorporation in growth-arrested rat mesangial cells stimulated with human PDGF-BB (10 and 50 ng/ml) and human PDGF-DD (10 to 200 ng/ml) with or without PDGF-B aptamer (100 ng/ml) or sequence scrambled aptamer control (100 ng/ml). Data are means ± SD of four independent experiments. *P < 0.05 versus unstimulated control.
Statistical Analyses

All values are expressed as means ± SD. Statistical significance (defined as \( P < 0.05 \)) was evaluated using ANOVA and Bonferroni \( t \) tests.

Results

**PDGF-DD Is a Mitogen for Mesangial Cells In Vitro**

Incubation of growth-arrested cultured rat mesangial cells with PDGF-DD led to a dose-dependent increase in proliferation (Figure 1). Independence of the mitogenic PDGF-DD activity from PDGF-BB was demonstrated by incubating the cells with antagonistic PDGF-BB aptamers or sequence-scrambled control aptamers simultaneously to PDGF-DD. Whereas the aptamers blocked PDGF-BB–induced proliferation, they had no effect on the mitogenic potential of PDGF-DD (Figure 1). Similar data were obtained with human mesangial cells (not shown).

**PDGF-DD Is Overexpressed in Glomeruli during Mesangioproliferative Nephritis**

After the induction of mesangioproliferative anti-Thy 1.1 nephritis in rats, glomerular PDGF-D mRNA expression initially decreased 0.4-fold at 4 h after disease induction but then increased to 2.4- and 2.9-fold at days 7 and 9, respectively, in comparison with nonnephritic rats (Figure 2). This latter peak paralleled that of glomerular PDGF-A mRNA expression and occurred with some delay after the maximum PDGF-B mRNA expression (Figure 2). In contrast to these three PDGF isoforms, PDGF-C mRNA was not upregulated during the first 28 d of anti-Thy 1.1 nephritis. To assess whether PDGF-D mRNA upregulation during anti-Thy 1.1 nephritis is specific for the kidney, we also investigated adrenal mRNA levels, because the adrenal gland has been noted to be a prominent source of PDGF-D (2). In contrast to glomeruli, no significant change in the PDGF-D mRNA expression level was observed in the adrenal glands during the first 28 d of anti-Thy 1.1 nephritis (data not shown). Despite these latter findings, a dramatic upregulation of PDGF-DD protein levels was detected in the serum of nephritic rats on day 8 after disease induction (27.7 ± 14.5 ng PDGF-DD/ml; \( n = 9 \)) compared with the levels in normal animals that were consistently below the detection limit (<0.02 ng/ml; \( n = 5 \); data not shown).

**Figure 2.** Transcript expression of PDGF-A, -B, -C, and -D in the course of anti-Thy 1.1 nephritis. Rats were killed at 4 h and at days 1, 2, 4, 7, 9, 14, 21, and 28 after disease induction (\( n = 5 \) each). RNA was isolated from the glomeruli, and the expression was measured by real-time quantitative PCR. The figure shows the transcript expression relative to the expression in untreated rats.

**Figure 3.** PDGF-DD is overexpressed during anti-Thy 1.1 nephritis in glomeruli. No PDGF-DD localization is noted in normal glomeruli (A), whereas mesangial expression can be readily detected during mesangioproliferative nephritis at day 7 after disease induction (B). No glomerular staining is present when the anti-PDGF-DD antibody is replaced by an equal concentration of control IgG (C). Magnification, ×600.
By immunohistochemistry, PDGF-DD localization in normal rat kidney was confined to arterial and arteriolar vascular smooth muscle cells, whereas no immunoreactivity was noted in glomeruli (Figure 3A). During anti-Thy 1.1 nephritis, prominent glomerular localization of PDGF-DD in the expanded mesangium was present (Figure 3B), whereas the remaining staining pattern of the kidneys was not affected.

Characterization of the Fully Human PDGF-DD mAb CR002.6.4

The specificity of fully human mAb CR002.6.4 for PDGF-DD among the PDGF was characterized by both solid-phase ELISA and Western blot analysis. As shown in Figure 4, A and B, mAb CR002.6.4 recognized human and murine PDGF-DD but not human PDGF-AA, PDGF-BB, or PDGF-CC. Control mAb PK16.3 showed no recognition of PDGF-DD. To confirm the ELISA result, Western blot analysis was also performed. Figure 4C shows that mAb CR002.6.4 immunoblotted human PDGF-DD (p35) but not PDGF-AA, PDGF-BB, or PDGF-CC. As demonstrated in Figure 4D, mAb CR002.6.4 also immunoblotted rat PDGF-DD in glomerular lysates of rats with anti-Thy 1.1 nephritis on day 8 after disease induction. The observed differences in the size of bands between human recombinant PDGF-DD (approximately 35 kD) and PDGF-DD in rat glomerular protein lysates (approximately 50 kD) potentially reflect differences in posttranslational modifications and/or processing differences. Control mAb PK16.3 recognized no PDGF. BIACore kinetic measurements were used to determine that the affinity of CR002.6.4 for human PDGF-DD was 170 pM and that CR002.6.4 had an approximately 20-fold lower affinity for murine PDGF-DD (data not shown).

CR002.6.4 was next tested for its ability to neutralize human PDGF-DD–induced mitogenic activity in a NIH 3T3 BrdU incorporation assay. As shown in Figure 5, CR002.6.4 neutralized human PDGF-DD–induced BrdU incorporation with an IC50 of approximately 75 ng/ml. PDGF-BB–induced BrdU incorporation was not affected at the highest concentrations tested (5 μg/ml; data not shown). Control mAb did not affect PDGF-DD–induced BrdU incorporation. CR002.6.4 also neutralized murine PDGF-DD–induced BrdU incorporation in normal human lung fibroblasts with an IC50 of approximately 250 ng/ml (data not shown). Concerning this, it is to stress that rat

Figure 4. Characterization of CR002.6.4 specificity by ELISA and Western blot analysis. (A and B) ELISA. Microtiter plates coated with human PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD (A) and with human or murine PDGF-DD (B) were incubated with the indicated concentration of PDGF-DD mAb CR002.6.4 or control mAb PK16.3 as described in the Materials and Methods section. (C and D) Western blot analysis. Nonreducing SDS-PAGE was performed after loading gels with 250 ng of human PDGF-AA, PDGF-BB, PDGF-CC, or PDGF-DD (C) or with 20 μg of rat glomerular protein lysates (D). After transfer to Hybond-P membranes, filters were treated with PDGF-DD mAb CR002.6.4 or control mAb PK16.3 as described in the Materials and Methods section.
and mouse PDGF-D are virtually identical. There is only one conservative amino acid substitution in the relevant core domain (L in rat to V in mouse) demonstrating homology of at least 99% between both species. This high homology and the specific recognition of rat PDGF-DD by CR002.6.4 (Figure 4D) strongly pled for a similar neutralization capacity of CR002.6.4 for rat PDGF-DD–induced mitogenic activity. Taken together, these results demonstrate that CR002.6.4 is highly specific for PDGF-DD, does not recognize other PDGF family members, and potently neutralizes PDGF-DD–induced BrdU incorporation.

**Inhibition of PDGF-DD In Vivo Reduces Pathologic Mesangial Cell Proliferation, Glomerular Matrix Accumulation, and Glomerular Monocyte/Macrophage Influx**

After the injection of anti-Thy 1.1 mAb, PBS-treated animals developed the typical course of nephritis, which is characterized by early mesangiolysis and is followed by a phase of mesangial cell proliferation and matrix accumulation on days 5 and 8. No obvious adverse effects were noted after the repeated injection of CR002.6.4 (all rats survived and seemed normal until the end of the study). Serum levels of the mAb that were achieved in the nephritic groups are shown in Table 2. Measurement of the total hemolytic activity (classic complement pathway) and hemolytic activity of the alternative complement pathway in rat sera with and without human mAb levels revealed normal hemolytic activity in all sera (CH50 < 284 kU/L; AP50 < 38% of normal range), suggesting no effects of the human IgG2 on complement fixation.

A considerable increase in albuminuria was present on day 7 in the nephritic as compared with nonnephritic rats (albumin/creatinine ratio, 15.5 ± 5.6 mg/μmol in nephritic rats receiving PBS versus 0.3 ± 0.1 mg/μmol in nonnephritic rats receiving control mAb PK16.3; P < 0.01). No significant differences were noted between the nephritic groups receiving PBS, control mAb PK16.3, or the three doses of CR002.6.4 (Table 2). CR002.6.4 did not induce proteinuria in normal rats. This absent effect of PDGF-DD antagonism on proteinuria in the anti-Thy 1.1 nephritis model is similar to observations made

Table 2. Human CR002.6.4 mAb or irrelevant control PK16.3 mAb levels achieved in vivo, urinary albumin/creatinine, and systolic BP

<table>
<thead>
<tr>
<th>Groups</th>
<th>Human mAb Serum Level (μg/ml) Day 5 after Disease Induction</th>
<th>Human mAb Serum Level (μg/ml) Day 8 after Disease Induction</th>
<th>Urinary Albumin/Creatinine Ratio (mg/μmol) Day 7 after Disease Induction</th>
<th>Systolic BP (mmHg) Day 7 after Disease Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephritic + CR002.6.4 5 mg/kg (day 3) + 2 mg/kg (day 5)</td>
<td>42 ± 9 (n = 7)</td>
<td>39 ± 26 (n = 7)</td>
<td>17.9 ± 9.4 (n = 7)</td>
<td>112 ± 11 (n = 3)</td>
</tr>
<tr>
<td>Nephritic + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)</td>
<td>75 ± 29 (n = 7)</td>
<td>65 ± 36 (n = 7)</td>
<td>18.0 ± 6.7 (n = 7)</td>
<td>136 ± 7 (n = 3)</td>
</tr>
<tr>
<td>Nephritic + CR002.6.4 20 mg/kg (day 3) + 8 mg/kg (day 5)</td>
<td>188 ± 85 (n = 8)</td>
<td>112 ± 72 (n = 8)</td>
<td>20.5 ± 23.3 (n = 8)</td>
<td>131 ± 21 (n = 4)</td>
</tr>
<tr>
<td>Nephritic + PK16.3 20 mg/kg (day 3) + 8 mg/kg (day 5)</td>
<td>134 ± 29 (n = 8)</td>
<td>95 ± 47 (n = 8)</td>
<td>15.7 ± 4.7 (n = 8)</td>
<td>119 ± 7 (n = 4)</td>
</tr>
<tr>
<td>Nephritic + PBS (day 3 and day 5)</td>
<td>&lt;0.02 (n = 9)</td>
<td>&lt;0.02 (n = 9)</td>
<td>15.5 ± 5.6 (n = 9)</td>
<td>132 ± 15 (n = 5)</td>
</tr>
<tr>
<td>Normal + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)</td>
<td>ND</td>
<td>ND</td>
<td>0.2 ± 0.3 (n = 5)</td>
<td>111 ± 11 (n = 3)</td>
</tr>
<tr>
<td>Normal + PK16.3 10 mg/kg (day 3) + 4 mg/kg (day 5)</td>
<td>ND</td>
<td>ND</td>
<td>0.3 ± 0.1 (n = 5)</td>
<td>122 ± 8 (n = 3)</td>
</tr>
</tbody>
</table>

* Data are mean values ± SD. ND, not determined.

Albumin/creatinine ratios in nephritic groups are not significantly different. Systolic BP are not significantly different between all treatment groups. Day of mAb treatment indicated in parentheses.
previously in the same model with a PDGF-B antagonist (22). No significant effects of the various CR002.6.4 doses or of irrelevant control mAb PK16.3 on systemic BP levels were observed, and all animals remained close to the normal range on day 7 (Table 2).

Glomerular cell proliferation, as assessed by counting the number of glomerular mitoses, was significantly reduced on day 8 in rats that received the anti–PDGF-D mAb CR002.6.4 as compared with rats that received control mAb PK16.3 or PBS (P < 0.001 for nephritic high-dose group versus nephritic controls; Figure 6). Counting of BrdU-positive nuclei confirmed these findings with the most pronounced suppression of proliferation on day 8 in the 20 + 8 mg/kg CR002.6.4-treated group (P < 0.001 for nephritic high-dose group versus nephritic controls; Figure 6).

To assess the treatment effects on mesangial cells, we immunostained the renal sections for α-smooth muscle actin, which is expressed only by activated mesangial cells (32). The glomerular expression of α-smooth muscle actin was significantly reduced on day 8 in the 10 + 4 mg/kg (P < 0.004) and 20 + 8 mg/kg (P < 0.005) CR002.6.4 mAb as compared with rats that received control PK16.3 mAb or PBS (Figure 6). To determine specifically whether mesangial cell proliferation was reduced, we double immunostained CR002.6.4 mAb, PK16.3 mAb, or PBS-treated rats for BrdU and α-smooth muscle actin. The data confirmed a marked decrease of proliferating mesangial cells on day 8 after disease induction in all three CR002.6.4-treated groups with a maximum of 57% reduction in mesangial cell proliferation (P < 0.009 for nephritic high dose group versus nephritic controls; Figure 6). Injection of CR002.6.4 into normal rats did not affect the physiologic glomerular cell turnover as compared with normal rats that received irrelevant IgG (Figure 6).

Interactions of PDGF-BB and PDGF-DD

Given that both PDGF-BB and PDGF-DD are overproduced in anti–Thy 1.1 nephritis (Figures 2 and 3) and given that antagonism of either results in a reduction of mesangioproliferative changes, we assessed potential interactions of the two PDGF isoforms. Antagonism of PDGF-DD with CR002.6.4 had no significant effect on glomerular PDGF-B and PDGF-D mRNA levels on day 8 of the disease (Table 3).

Discussion

In this study, PDGF-DD was shown to induce proliferation in cultured rat and human mesangial cells. This observation is
consistent with previous data showing that mesangial cells in
culture express both the PDGF receptor α- and β-chain (12).
Furthermore, this study demonstrated intraglomerular overpro-
duction and overexpression of PDGF-D-chain during the
course of experimental mesangioproliferative nephritis. In con-
trast to most other cytokines and growth factors that act in a
localized auto- or paracrine manner, PDGF-D in this model
was notable for its dramatic increase in serum, i.e., potential
endocrine effects. In normal rat glomeruli, PDGF-D expression
was absent, similar to recent findings on the expression of
PDGF-C in rat kidney (7). In this respect, the species differ-
cences between rat and human kidney is notable, because both
PDGF-C and -D are expressed in normal human podocytes,
i.e., glomerular visceral epithelial cells (6,7).

Given the above findings, we asked whether inhibition of
PDGF-DD in the anti-Thy 1.1 mesangioproliferative nephritis
model might result in a reduction of mesangioproliferative
changes in vivo. In the anti-Thy 1.1 nephritis model, immu-
nologic damage to the mesangium results in mesangiolysis,
duces migration of nondamaged mesangial cells from the
extraglomerular mesangium, and results in excessive me-
sangioproliferation leading to mesangioproliferative glomerulonephritis (38).
In the present study, as in previous ones (22,25), we therefore
initiated treatment on day 3 to avoid any interference of the
therapy with disease induction, e.g., glomerular binding of the
nephritogenic antibody, complement activation, cytotoxic
damage and mesangiolysis, all of which peak within 24 to 48 h
of disease initiation. This timing was also chosen on the basis
of glomerular PDGF-D mRNA kinetics that revealed an in-
crease in PDGF-DD between days 2 and 4. Finally, this ex-
perimental design mimicked the clinical setting because treat-
ment was initiated after the onset of mesangioproliferative
glomerulonephritis.

Pharmacologic intervention with neutralizing PDGF-D mAb
CR002.6.4 on days 3 and 5 after induction of anti-Thy 1.1
nephritis resulted in a significant dose-dependent reduction of
excessive mesangial cell proliferation and thereby led to a
marked reduction of glomerular hypercellularity. By double
immunostaining, we were able to demonstrate that reduction of
mesangial cell proliferation is a central contributor to the
therapeutic effects observed. It seems unlikely that this was a
nonspecific effect, for example related to hemodynamic
changes or effects on disease induction because CR002.6.4
mAb had no effect on systemic BP or mesangiolysis. Inciden-
tially, the absent effect of the antibody on mesangiolysis addi-
tionally supports the idea of PDGF-D being a secreted rather
than a membrane-bound protein (36) because enhanced me-
sangiolysis would have been the likely consequence if mAb
CR002.6.4 were to bind to mesangial cells. The latter is also
unlikely, because we failed to detect mesangial deposition of
human IgG in rats that received the antibody. Finally, reduction
of mesangial cell proliferation was specific for the CR002.6.4
mAb treatment.

Of further interest is whether CR002.6.4 mAb treatment
directly reduced mesangial cell proliferation in vivo or, for
example, via effects on other growth factors. Such cascades
seem to be operative in anti-Thy 1.1 nephritis, as we have
recently demonstrated that PDGF-BB most likely is a down-
stream mediator of TGF-β effects in vivo (39). A comparative
evaluation of the mRNA expression of all four PDGF isoforms
during anti-Thy 1.1 nephritis revealed that the peak of
PDGF-D mRNA overproduction occurred after that of
PDGF-B. Whether this is evidence of an induction of PDGF-D
by PDGF-B in vivo remains unknown.

It is interesting that the glomerular fibronectin accumulation
on day 8 was significantly reduced by PDGF-DD antagonism
in the two high-dose groups, whereas glomerular type I colla-
gen expression remained unchanged. These results point to-
dard differences between PDGF-DD and PDGF-BB regulation
of the glomerular matrix protein turnover because it has been
demonstrated that PDGF-BB antagonism in experimental me-
sangioproliferative glomerulonephritis resulted in a significant
reduction of both glomerular fibronectin and type I collagen
(22).

In addition to selectively abrogating mesangial cell prolif-
eration, treatment with the CR002.6.4 mAb, at least with
higher doses, reduced the glomerular monocyte/macrophage
influx on day 5. Whether this is affected directly or through
modulation of other mediators is unknown. In this context, it
seems noteworthy that monocyte chemoattractant protein-1
expression, which is known to be responsible for the glomer-
ular infiltration of monocytes/macrophages in the early phase

Table 3. Glomerular PDGF-B and PDGF-D mRNA expression in CR002.6.4-treated rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>PDGF-B mRNA (Relative to Expression in Normal Rats + Control PK16.3)</th>
<th>PDGF-D mRNA (Relative to Expression in Normal Rats + Control PK16.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephritic + CR002.6.4 5 mg/kg (day 3) + 2 mg/kg (day 5)</td>
<td>1.60 (1.3–1.9) (n = 2)</td>
<td>1.90 (1.5–2.2) (n = 2)</td>
</tr>
<tr>
<td>Nephritic + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)</td>
<td>1.25 (1.0–1.6) (n = 3)</td>
<td>1.40 (1.2–1.5) (n = 3)</td>
</tr>
<tr>
<td>Nephritic + CR002.6.4 20 mg/kg (day 3) + 8 mg/kg (day 5)</td>
<td>1.35 (1.1–1.5) (n = 2)</td>
<td>1.45 (1.2–1.7) (n = 2)</td>
</tr>
<tr>
<td>Nephritic + PK16.3 20 mg/kg (day 3) + 8 mg/kg (day 5)</td>
<td>1.40 (1.1–1.7) (n = 3)</td>
<td>1.60 (1.4–1.7) (n = 3)</td>
</tr>
<tr>
<td>Nephritic + PBS (day 3 and day 5)</td>
<td>1.45 (1.1–1.8) (n = 3)</td>
<td>2.10 (1.5–2.6) (n = 3)</td>
</tr>
<tr>
<td>Normal + CR002.6.4 10 mg/kg (day 3) + 4 mg/kg (day 5)</td>
<td>0.95 (0.7–1.1) (n = 2)</td>
<td>1.10 (1.0–1.2) (n = 2)</td>
</tr>
<tr>
<td>Normal + PK16.3 10 mg/kg (day 3) + 4 mg/kg (day 5)</td>
<td>1.0 (n = 2)</td>
<td>1.0 (n = 2)</td>
</tr>
</tbody>
</table>

*Data are means (and ranges) of pooled fractions within each treatment group. Measurements were performed twice for each sample.
Day of mAb treatment indicated in parentheses.
of the anti-Thy 1.1 nephritis (40), is rapidly induced in fibroblasts upon stimulation with PDGF-BB (41).

In conclusion, this study provides the first evidence to implicate glomerular and systemic overexpression of PDGF-DD in the pathogenesis of mesangioproliferative changes, in particular enhanced mesangial cell proliferation. Thus, we demonstrate that PDGF-DD exerts the biologic effect in vitro and that the biologic effect in vivo is associated with overproduction and release of the factor. In addition, the in vivo effect can be diminished by specific antagonism of PDGF-DD. We thereby fulfill three of four criteria necessary for establishing a role for a growth factor in glomerular disease (42). The fourth criterion, namely “the effect is reproduced in vivo by administration or overexpression of the factor,” was recently reported in preliminary fashion by Hudkins et al. (43).

Acknowledgments

This study was supported by a grant (SFB 542/C7) of the Deutsche Forschungsgemeinschaft.

The technical help of Gertrud Minartz, Gabriele Dietzel, Bill McDonald, and Andrea Cosler is gratefully acknowledged.

References


