Antagonism of PDGF-D by Human Antibody CR002 Prevents Renal Scarring in Experimental Glomerulonephritis

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Glomerular mesangial cell proliferation and/or matrix accumulation characterizes many progressive renal diseases. PDGF-D was identified recently as a novel mediator of mesangial cell proliferation *in vitro* and *in vivo*. This study investigated the long-term consequences of PDGF-D inhibition *in vivo*. Rats with progressive mesangioproliferative glomerulonephritis (uninephrectomy plus anti–Thy-1.1 antibody) received the PDGF-D–neutralizing, fully human mAb CR002 on days 3, 10, and 17 after disease induction. Glomerular mesangioproliferative changes on day 10 were significantly reduced by anti–PDGF-D treatment as compared with control antibody. Eight weeks after disease induction, anti–PDGF-D therapy significantly ameliorated focal segmental glomerulosclerosis, podocyte damage (*de novo* desmin expression), tubulointerstitial damage, and fibrosis as well as the accumulation of renal interstitial matrix including type III collagen and fibronectin. Treatment with anti–PDGF-D also reduced the cortical infiltration of monocytes/macrophages on day 56, possibly related to lower renal cortical complement activation (C5b-9 deposition) and/or reduced epithelial-to-mesenchymal transition (preserved cortical expression of E-cadherin and reduced expression of vimentin and α-smooth muscle actin). In conclusion, these data provide evidence for a causal role of PDGF-D in the pathogenesis of renal scarring and point to a new therapeutic approach to progressive mesangioproliferative renal disease.

The number of patients with ESRD continues to grow in most Western countries (1). Besides diabetic nephropathy, which underlies up to 35% of ESRD cases, glomerulonephritides, in particular the most common type IgA nephropathy, account for approximately 20% of ESRD cases in most Western countries. Both diabetic nephropathy and the majority of progressive glomerulonephritides are histologically characterized by glomerular mesangial cell proliferation and/or matrix accumulation (2,3). Ample evidence now is available to link the PDGF system to these processes. The role of PDGF-B chain in mediating mesangioproliferative changes is well established (4–6), but the role of PDGF-D has been elucidated only recently (7–10). Like PDGF-B, PDGF-D signals through the PDGF-β receptor (7,8). PDGF-B binds to all three known PDGF receptors with high affinity (11); in contrast, PDGF-D binds predominantly to the PDGF-β receptor (7,8). This suggests that PDGF-B, in comparison with PDGF-D, may signal through at least partially different pathways.

Several recent studies link PDGF-D to renal disease. PDGF-D induces mesangial cell proliferation *in vitro* and is overexpressed in mesangioproliferative glomerulonephritis *in vivo* (9).

Adenoviral expression of PDGF-D in the liver induced prominent mesangioproliferative nephritis in mice (10). PDGF-β receptor knockout mice fail to develop a mesangium (12), and, finally, specific antagonism of PDGF-D chain with the fully human mAb CR002 reduced mesangial cell proliferation and matrix accumulation in a (reversible, nonprogressive) rat model of mesangioproliferative nephritis, the anti–Thy-1.1 model (9).

On the basis of these observations, specific antagonism of PDGF-D might represent a novel therapeutic approach to progressive renal diseases that are characterized by mesangial expansion. However, the only intervention study so far has been confined to short-term observations (9). Consequently, it is unproved whether antagonism of PDGF-D in glomerular disease exerts long-term beneficial effects. In this study, we investigated the long-term consequences of PDGF-D antagonism using a progressive anti–Thy-1.1 nephritis model similar to the model of Cheng *et al.* (13). This model is induced by uninephrectomy and anti–Thy-1.1 antibody administration and, when left untreated, progresses to renal failure.

Materials and Methods

Fully Human PDGF-D MAb CR002

Generation and specificity of fully human PDGF-DD mAb CR002 was described in detail previously (9,14).

Experimental Model and Experimental Design

All animal studies were approved by the local review boards. Male Wistar rats (*n* = 29) that weighed 180 g were purchased from Charles

Table 1. Human mAb levels achieved *in vivo* on days 16, 28, and 56 after disease induction as well as ELISA data on rat anti-human IgG in serum on day 56[a]

Groups	Human mAb Serum Level (μg/ml), Day 16	Human mAb Serum Level (μg/ml), Day 28	Human mAb Serum Level (μg/ml), Day 56	Rat Anti-Human IgG (AU), Day 56
Nephritic + IgG$_2$ ($n = 9$)	10 ± 21	1 ± 0.7	0.3 ± 0.1	155 ± 227
Nephritic + CR002 ($n = 10$)	2 ± 0.6	5 ± 3	1 ± 1	110 ± 113
Nonnephritic + IgG$_2$ ($n = 5$)	153 ± 46	34 ± 25	19 ± 34	162 ± 182
Nonnephritic + CR002 ($n = 5$)	123 ± 25	144 ± 61	22 ± 14	76 ± 80
Nephritic + PBS ($n = 10$)	ND	ND	ND	202 ± 148[b]

[a]Data are arbitrary optical units per 10 μl of serum; all differences between groups are not significant by ANOVA and Bonferroni *t* tests. AU, arbitrary units; ND, not determined.
[b]Four sera in the PBS group were taken on days 26, 34, 35, and 43 at the time of death of the animals.

River (Sulzfeld, Germany). Progressive anti–Thy-1.1 nephritis was induced by a right-sided uninephrectomy followed 1 h later with a single intravenous bolus injection of 1 mg/kg anti–Thy-1.1 mAb (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, England). Rats were treated with the anti–PDGF-D mAb CR002 or control IgG$_2$ (Fitzgerald Industries International Inc., Concord, MA) on days 3, 10, and 17 after disease induction. Treatment consisted of intraperitoneal injections of the antibodies dissolved in 800 μl of 20 mM Tris-HCl/100 mM NaCl (pH 7.4). The timing of antibody treatment was chosen to target mesangial cell proliferation, which occurs between days 5 and 10 after disease induction. CR002 and control IgG$_2$ serum levels were measured on days 16, 28, and 56 (Table 1).

Four groups of uninephrectomized rats were studied:

1. Nephritic + control IgG ($n = 9$): 1 mg/kg body wt control IgG$_2$
2. Nephritic + CR002 ($n = 10$): 1 mg/kg body wt CR002
3. Nonnephritic + control IgG ($n = 5$)
4. Nonnephritic + CR002 IgG ($n = 5$)

The antibody dose in the nephritic groups was selected because higher doses induced complement depletion, making interpretation of the results too problematic (data not shown). Food, in particular protein intake, in all groups was kept at similar levels by pair feeding the rats. Intravital renal biopsies were obtained on day 10 from five randomly selected rats each of groups 1 and 2 for histologic evaluation (15). Approximately 400 μl of venous blood was drawn weekly from a tail vein, and 24-h urine collections were performed weekly until the rats were killed on day 56. BP was measured by tail-cuff plethysmography on days 2, 28, and 55. After the rats were killed, serum samples and renal tissues for histologic evaluation were collected. The remaining cortical tissue of each rat was used to isolate RNA (see below).

Cortical RNA Extraction and Analyses

Total RNA was extracted from renal cortex using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA syntheses and real-time quantitative PCR were performed as described previously (9). Sequences of primers and probes that were used in this study are listed in Table 2. mRNA for each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase.

Renal Morphology

Tissue for light microscopy and immunoperoxidase staining was fixed in methyl Carnoy's solution as well as in formalin and embedded in paraffin. Four-micrometer sections were stained with the periodic acid-Schiff reagent and counterstained with hematoxylin. In these sections of day 10 biopsies, the number of mitoses within 50 to 100 glomerular cross-sections of consecutive cortical glomeruli each, which contained >20 discrete capillary segments, was evaluated. On day 56, the percentage of glomeruli that exhibited focal or global glomerulosclerosis was determined as described (16). Tubulointerstitial injury on day 56 was graded according to Shih *et al.* (17) on a scale of 0 to 4 as described previously (6).

For the evaluation of total collagen, renal tissues were stained with Sirius red and evaluated by computer-based morphometry (Soft Imaging System GmbH, Münster, Germany). The percentage of positively stained area in each tissue was calculated in 20 fields, with each field having an area of 0.37 mm^2. In all analyses, the investigator was unaware of the origin of the slides.

Immunoperoxidase Staining

Four-micrometer sections of methyl Carnoy's–fixed biopsy tissue were processed as described previously (16). Primary antibodies included a murine mAb (clone 1A4) to α-smooth muscle actin (α-SMA); a murine monoclonal IgG antibody (clone ED1) to monocytes, macrophages, and dendritic cells; a murine mAb (clone D33) against human muscle desmin; affinity-purified polyclonal goat anti-human/bovine type IV collagen IgG; affinity-purified polyclonal goat antibodies against human types I and III collagen (Southern Biotechnology, Birmingham, AL); an affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA); a murine mAb

Table 2. Primers and probes[a]

Gene (Rat)	Forward Primer	Reverse Primer	Taqman Probe	GenBank Accession No.
GAPDH	5′-ACAAGATGGTGAAGGTCGGTG-3′	5′-AGAAGGCAGCCCTGGTAACC-3′	5′-CGGATTTGGCCGTATCGGACGC-3′	AF106860
Fibronectin	5′-AAACAGGTCTGGACTCCCCA-3′	5′-CAGAATGCTCGGCGTGATG-3′	5′-TCTTCTGATGTCACCGCCAACTCATTCA-3′	X15906
Type I collagen	5′-GAAGGCAACAGTCGATTCACC-3′	5′-GACTGTCTTGCCCCAAGTTCC-3′	5′-ACAGCACGCTTGTGGATGGCTGC-3′	Z78279

[a]GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

(clone V9; DAKO, Glostrup, Denmark) against porcine vimentin; a murine monoclonal IgG against human E-cadherin (DakoCytomation, Glostrup, Denmark); and affinity-purified polyclonal biotinylated donkey antibody against human IgG (Dianova, Hamburg, Germany), plus appropriate negative controls as described previously (18,19).

To evaluate complement C5b-9 deposition, we incubated formalin-fixed, paraffin-embedded tissues with protease type XXIV (Sigma Aldrich Chemie GmbH, Steinheim, Germany), 0.4 mg/ml, for 10 min at 37°C followed by a murine mAb (clone 2A1; a gift of S. Shankland, University of Washington, Seattle, WA) against rat C5b-9 (20).

For the evaluation of the staining for glomerular α-SMA and type IV collagen on day 10, each glomerular area was graded semiquantitatively, and the mean score per biopsy was calculated as described previously (15). For obtaining total counts of infiltrating monocytes/macrophages in the renal interstitium, >20 to 30 grid fields (magnification 100-fold) that measured 0.09 mm² each were analyzed and mean counts per kidney were obtained. The stains for cortical types I and III collagen, fibronectin, E-cadherin, and C5b-9 were evaluated by computer-based morphometry. The percentage of staining in each field was calculated in 20 fields per tissue (magnification 100-fold, measuring 0.37 mm² per field). The expression of tubulointerstitial α-SMA, vimentin, and desmin was scored semiquantitatively because the staining was too focal to allow reliable morphometric assessments. Vessels, which constitutively expressed these markers, were not counted. Staining was scored on the extent of interstitial foci: 0, normal pattern; 0.5, very rare, trace interstitial foci; 1, <5% of cortex exhibiting locally increased staining; 2, 5 to 10%; 3, 10 to 20%; and 4, 20 to 30% of cortex exhibiting positive staining. In the case of immunostaining for desmin, edges of glomerular tufts, reflecting podocyte de novo expression of desmin, also were scored semiquantitatively as described previously (6).

Serum Measurements of mAb CR002 and Rat Anti-Human IgG

Circulating levels of anti–PDGF-DD (CR002) mAb were measured by ELISA. Briefly, 96-well ELISA plates (Corning, Acton, MA) were coated with 1 µg/well recombinant PDGF-D in bicarbonate buffer (pH 9.6) at 4°C overnight. After washing, plates were blocked with 200 µl/well blocking buffer (Pharmingen Diluent; BD Pharmingen, San Diego, CA) for 1 h and washed, and 100 µl/well sample or standard was added to each well. CR002 in rat serum served as a standard. Plates were incubated at room temperature for 1 h and washed, and goat anti-human IgG κ coupled to horseradish peroxidase (HRP) and diluted in PBS that contained 0.5% BSA and 0.1% Tween-20 was added to each well at 100 µl/well. After the plates were washed, 100 µl/well substrate was added (BD Pharmingen TMB reagent A&B), and the plates incubated for 5 min in the dark. The reaction was stopped by the addition of 100 µl/well 1 N HCL, and the optical density was determined at 450 nm using a microplate reader (TECAN; Research Triangle Institute, Research Triangle Park, NC). Concentrations of CR002 in serum samples were calculated using Softmax Pro software (Molecular Devices, Sunnyvale, CA).

For assessment of whether CR002 treatment induced the formation of rat anti-human IgG, ELISA plates were coated with 0.22 µg/well human IgG (Dianova) in PBS at 4°C overnight. After washing and blocking with 2% BSA/PBS-Tween (0.05%) for 1 h, wells were incubated with 10 µl of rat serum in PBS for 1 h. Subsequently, plates were washed and incubated with biotinylated goat anti-rat IgG (2 mg/ml, 1:10,000; Dianova), followed by washing and by incubation with streptavidin-coupled HRP for 1 h. After washing, substrate solution that contained 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic-acid; Sigma, Taufkirchen, Germany) was added to the wells for 30 min, and optical density was determined at 405 nm in a microplate reader (Dynatech, Chantilly, VA). The optical densities were compared with values that were obtained in wells with sera of healthy, nontreated rats. As a control, rat anti-human IgG also was measured in the

sera of an extra group of nephritic rats ($n = 10$) that were treated on days 3, 10, and 17 with PBS only.

Miscellaneous Measurements

Urinary albumin levels were determined by ELISA (Nephrat; Exocell, Philadelphia, PA). Serum creatinine was determined by an autoanalyzer. BP measurements were performed by the tail-cuff method, using a programmed sphygmomanometer (Softron Co., Tokyo, Japan).

Urinary levels of α-1-microglobulin were measured by Western blot: Rat urine (1 µg protein/lane) was loaded and separated by SDS-PAGE using 4% acrylamide for the stacking and 12.5% for the resolving gel. Protein was transferred to nitrocellulose membranes (pore size 0.45 µm; Schleicher & Schuell, Keene, NH) and probed with a polyclonal antibody against rat α-1-microglobulin (rabbit polyclonal antibody; AgriSera, Vännäs, Sweden). The primary antibody was detected using HRP-conjugated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA). The negative control consisted of substitution of the primary antibody with equivalent concentrations of normal rabbit IgG. Bound antibodies were visualized by the ECL system (Amersham Pharmacia Biotech, Freiburg, Germany). The intensity of the identified 27-kD band was quantified by densitometry using the system of Biostep GmbH (Jahnsdorf, Germany), corrected for the relative intensity of a nonspecific band and subsequently calculated to correspond to 1 nmol of urinary creatinine in the samples. The analysis software was from TotalLab (Phoretix International, Newcastle, UK).

Statistical Analyses

All values are expressed as means ± SD. Statistical significance (defined as $P < 0.05$) was evaluated using ANOVA and Bonferroni t test or paired t test where appropriate.

Results

Acute Effects of PDGF-D Antagonism in Rats with Progressive Glomerulonephritis

On day 10 after disease induction, treatment with CR002 significantly reduced the number of glomerular mitoses in comparison with the control antibody (Figure 1A). CR002 significantly ameliorated the glomerular de novo expression of α-SMA (Figure 1B), a specific marker of mesangial cell activation (21).

Chronic Effects of PDGF-D Antibody CR002 in Rats with Progressive Glomerulonephritis

Albuminuria, Renal Function, BP, and Body Weight. As shown in Table 3, albuminuria on days 49 and 56 after disease
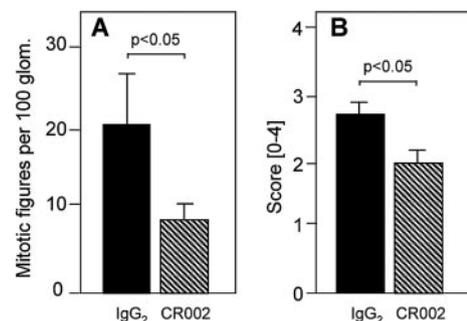

Figure 1. Glomerular frequency of mitotic figures (A) and expression of α-smooth muscle actin (α-SMA; B) on day 10 after disease induction in rats that received either PDGF-D antibody CR002 or irrelevant IgG₂ on day 3 ($n = 5$ per group).

Table 3. Effects of PDGF-D antibody CR002 treatment on serum creatinine and albuminuria

Groups	Day 49 after Disease Induction		Day 56 after Disease Induction	
	Serum Creatinine (μmol/L)	Albuminuria (mg/24 h)	Serum Creatinine (μmol/L)	Albuminuria (mg/24 h)
Nephritic + IgG$_2$ ($n = 9$)	41 ± 3	8 ± 10	37 ± 4	7 ± 10
Nephritic + CR002 ($n = 10$)	34 ± 3^a	4 ± 5	34 ± 4	7 ± 10
Nonnephritic + IgG$_2$ ($n = 5$)	34 ± 2^a	0.2 ± 0.1	35 ± 2	0.3 ± 0.2
Nonnephritic + CR002 ($n = 5$)	35 ± 3^a	0.4 ± 0.4	33 ± 5	0.7 ± 0.9

[a]$P < 0.05$ *versus* nephritic rats that received IgG$_2$.

Table 4. Effects of PDGF-D antibody CR002 treatment on systolic BP and body weight[a]

Groups	Systolic BP (mmHg) Day 2	Systolic BP (mmHg) Day 28	Systolic BP (mmHg) Day 55	Body Weight (g) Day 56
Nephritic + IgG$_2$	102 ± 11 ($n = 5$)	120 ± 8 ($n = 6$)	134 ± 15^b ($n = 7$)	376 ± 34 ($n = 9$)
Nephritic + CR002	118 ± 6 ($n = 2$)	115 ± 14 ($n = 5$)	132 ± 14 ($n = 7$)	402 ± 41 ($n = 10$)
Nonnephritic + IgG$_2$	108 ± 1.7 ($n = 2$)	118 ± 11 ($n = 5$)	127 ± 5 ($n = 5$)	422 ± 14 ($n = 5$)
Nonnephritic + CR002	104 ± 3 ($n = 3$)	110 ± 15 ($n = 5$)	125 ± 9 ($n = 5$)	410 ± 68 ($n = 5$)

[a]All BP and body weight differences between treatment groups at each time point (ANOVA and Bonferroni *t* test) are NS.
[b]Statistically significant difference ($P < 0.05$, paired *t* test) within a treatment group between BP on days 2 and 55.

induction was 10- to 40-fold higher in nephritic rats as compared with uninephrectomized, nonnephritic rats. Treatment of nephritic rats with the neutralizing PDGF-D antibody on days 3, 10, and 17 tended to reduce albuminuria on day 49 but not on day 56 after disease induction, compared with the nephritic group that was treated with irrelevant IgG. However, variability within groups was high on day 49, and differences between groups failed to reach significance. Albuminuria at earlier time points was not different between both nephritic groups. Assessment of total proteinuria yielded very similar findings (data not shown). Excretion of a low molecular weight protein, α-1-microglobulin, in the urine of nephritic rats on day 56 after disease induction was similar in all groups and not significantly different from that observed in normal rats (data not shown).

Serum creatinine concentrations on days 49 and 56 increased in nephritic rats that received control IgG in comparison with nonnephritic rats (Table 3). In contrast, creatinine levels on days 49 and 56 were not different from those of nonnephritic rats in the group that was treated with neutralizing PDGF-D antibody (Table 3).

BP were normal in all rats on day 2 after disease induction and increased thereafter until day 55, with a significant increase within the nephritic control IgG–treated group (Table 4). Treatment with CR002 did not affect BP levels (Table 4).

Despite pair feeding, nephritic control rats on day 56 tended to exhibit lower body weights than nonnephritic rats, suggesting increased catabolism in the former groups. Treatment with CR002 led to higher, albeit not normalized, body weights (Table 4).

Glomerulosclerosis and Tubulointerstitial Damage. By day 56, nephritic rats that were treated with control IgG had developed widespread glomerulosclerosis (Figure 2A). Treatment with CR002 markedly reduced the frequency of sclerotic glomeruli (Figure 2, B and C). Comparison of the serum levels of CR002 (Table 1) and the percentage of sclerotic glomeruli in

each animal showed a clear correlation between the level of CR002 exposure at day 56 and glomerular damage ($P = 0.027$). To assess more specifically podocyte damage, which occurs
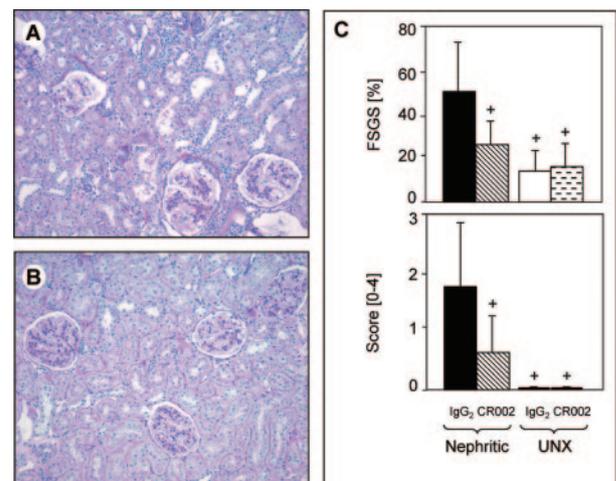

Figure 2. Renal morphologic changes on day 56 after disease induction. (A and B) Periodic acid-Schiff–stained renal section of a nephritic rat that received control IgG$_2$, showing widespread glomerulosclerosis and focal tubular atrophy, interstitial infiltration, and fibrosis (A), or a rat that received CR002 on days 3, 10, and 17, showing some residual mesangial expansion, occasional glomerulosclerosis and no tubulointerstitial damage (B). (C) Frequency of focal segmental glomerulosclerosis (FSGS) and tubulointerstitial damage scores. Data are means $\pm$ SD. [+]$P < 0.05$ *versus* nephritic rats that received control IgG$_2$. Nephritic, rats with anti–Thy-1.1 nephritis after previous uninephrectomy; UNX, nonnephritic rats that had received a uninephrectomy only. Magnification, $\times 100$.

secondary to mesangial injury in anti–Thy-1.1 nephritis (6), we studied renal sections for the *de novo* expression of desmin, a nonspecific injury marker in podocytes (22). As shown in Figure 3, nephritic animals that received control IgG showed desmin at the edge of the glomerular tuft on average in 50% of the glomerular circumference. Treatment with CR002 led to a significant reduction of staining scores in comparison with the nephritic control (Figure 3).

Nephritic rats that received control IgG exhibited tubulointerstitial damage that affected on average 10 to 25% of the cortex (Figure 2C). Treatment with CR002 led to a significant reduction of scores in comparison with the nephritic group that was treated with control IgG (Figure 2C). Renal interstitial fibrosis also was assessed by Sirius red staining. Figure 4 shows that PDGF-D antagonism significantly reduced renal interstitial fibrosis in comparison with nephritic rats that had received control IgG only.

The development of interstitial fibrosis was studied further by investigation of the expression of specific matrix proteins. As shown in Figure 5A, nephritic rats that received control IgG showed interstitial foci of type III collagen accumulation, involving approximately 5% of the renal cortex. Treatment with CR002 significantly reduced accumulation of type III collagen in comparison with the nephritic control (Figure 5, B and C). Similarly, renal interstitial accumulation of type I collagen was reduced by an average of 30 to 40% in the CR002-treated nephritic group as compared with the nephritic control (data not shown). Finally, the tubulointerstitial accumulation of fibronectin was significantly reduced in CR002-treated nephritic rats as compared with the nephritic control (Figure 5D). Quantification of renal cortical mRNA levels for type I collagen and fibronectin failed to reveal significant differences between the groups (data not shown).

Tubulointerstitial Complement Activation and Epithelial-to-Mesenchymal Transition. To assess the mechanisms that are involved in reduced tubulointerstitial damage after PDGF-D antagonism, we investigated tubulointerstitial complement activation, which mediates most of the "toxicity" of proteinuria (23), and epithelial-to-mesenchymal transition (EMT) (24). During EMT, renal tubular cells lose expression of
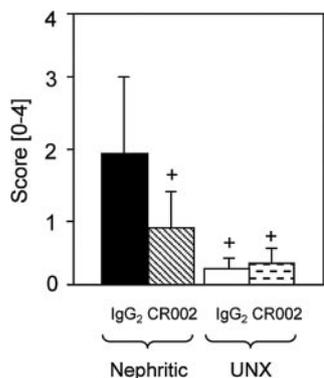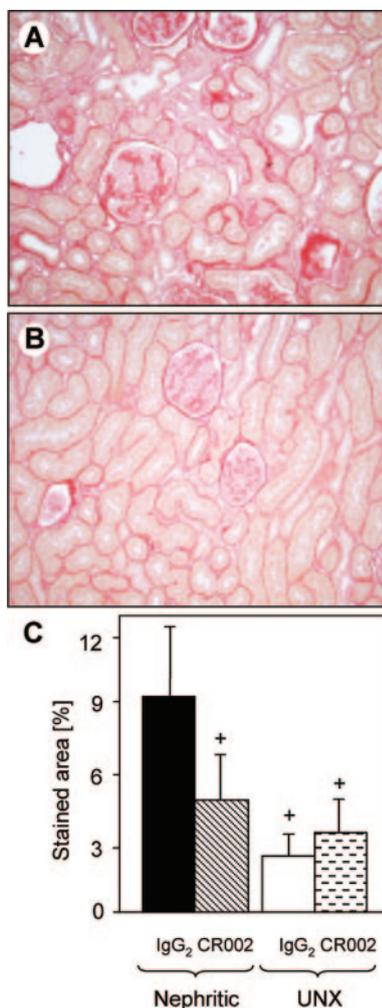

Figure 4. Renal interstitial fibrosis as detected by Sirius red staining. (A and B) Section of a nephritic rat that received control IgG$_2$, showing widespread foci of interstitial fibrosis associated with tubular atrophy (A), or a rat that received CR002 on days 3, 10, and 17, showing rare interstitial expansion only (B). (C) Morphometric evaluation of Sirius red–positive area in the renal cortex. Data are means ± SD. $^+P < 0.05$ *versus* nephritic rats that received control IgG$_2$. Magnification, ×100.

Figure 3. Expression of desmin at the glomerular edge on day 56 after disease induction. Data are means ± SD. $^+P < 0.05$ *versus* nephritic rats that received control IgG$_2$.

tubular markers, such as E-cadherin, and start to express markers of (myo-)fibroblasts, such as vimentin or α-SMA (24–26). As illustrated in Figure 6, treatment of rats with CR002 significantly reduced tubulointerstitial deposition of C5b-9 in comparison with nephritic control rats.

Staining of kidneys for E-cadherin revealed a >50% decrease in the expression of E-cadherin on the basolateral part of tubular epithelial cells in nephritic control rats as compared with nonnephritic rats (Figure 7, A, B, and D). Previous treatment with CR002 largely restored the normal expression pattern in nephritic rats (Figure 7, C and D). In the case of vimentin, increased tubular and tubulointerstitial expression was noted in the cortex of nephritic control rats compared with nonnephritic rats (Figure 7, E, F, and H). CR002 treatment led to a significant reduction of tubulointerstitial vimentin expression on day 56 in the group of nephritic rats (Figure 7, F, G, and H).
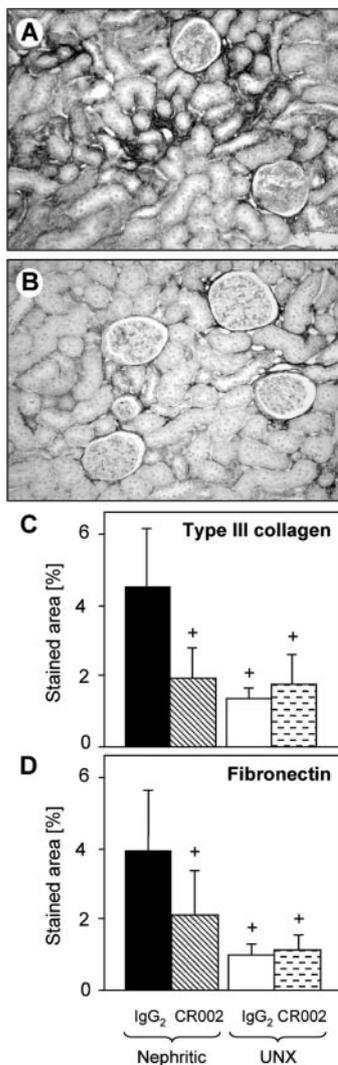
Figure 5. Renal cortical deposition of type III collagen. (A and B) Section of a nephritic rat that received control IgG$_2$, showing widespread foci of collagen accumulation (A), or a rat that received CR002 on days 3, 10, and 17, showing low-grade accumulation of type III collagen in the interstitium (B). (C) Morphometric evaluation of the area that stained positively for type III collagen in the renal cortex. (D) Morphometric evaluation of the area that stained positively for fibronectin in the renal cortex. Data are means ± SD. $^+P < 0.05$ *versus* nephritic rats that received control IgG$_2$. Magnification, ×100.

Finally, increased expression of tubulointerstitial α-SMA and desmin in the nephritic control group was reduced by CR002 treatment, albeit not significantly for desmin (data not shown).

Renal Monocyte/Macrophage Infiltration. Tubulointerstitial monocyte/macrophage counts increased more than two-fold in nephritic control rats as compared with nonnephritic rats on day 56 after disease induction (Figure 8). Treatment of nephritic rats with CR002 led to a significant reduction of tubulointerstitial monocyte/macrophage infiltration in comparison with nephritic control rats (Figure 8).

Deposition of CR002 in Renal Tissue and Formation of Rat Anti-Human IgG Antibody. As shown in Table 1, no rat anti-human IgG, which might have neutralized the activity of
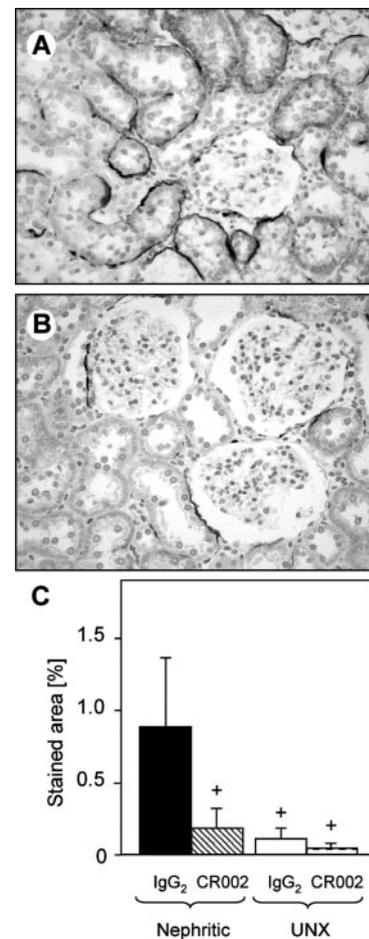

Figure 6. Renal interstitial detection of membrane attack complex (C5b-9). (A and B) Section of a nephritic rat that received control IgG$_2$, showing widespread C5b-9 complex formation at the glomerular and tubular epithelium (A), or a rat that received CR002 on days 3, 10, and 17, showing only occasional C5b-9 complex formation at both epithelia (B). (C) Morphometric evaluation of C5b-9–positive area in the renal cortex. Data are means ± SD. $^+P < 0.05$ *versus* nephritic rats that received control IgG$_2$. Magnification, ×100.

CR002, was detectable in any group. All values were below those of a nephritic group that was treated with PBS only. Furthermore, no intrarenal deposition of human IgG was noted in any group on day 56 (data not shown).

Discussion

In this study, we extend our previous work, which showed that specific antagonism of PDGF-D in rat mesangioproliferative glomerulonephritis reduces mesangial cell proliferation, glomerular influx of monocytes/macrophages, and fibronectin accumulation (9). This first study on PDGF-D in renal disease was confined to a model of acute damage, which is not suitable to study long-term sequelae of PDGF-D antagonism, as spontaneous healing of the glomerular changes occurs in "standard" anti–Thy-1.1 nephritis independent of treatment (27). We therefore aggravated anti–Thy-1.1 nephritis by performing a uninephrectomy before disease induction. Using the monoclonal

Figure 8. Tubulointerstitial influx of monocytes/macrophages on day 56 after disease induction. Data are means ± SD. [+]$P <$ 0.05 *versus* nephritic rats that received control IgG$_2$.
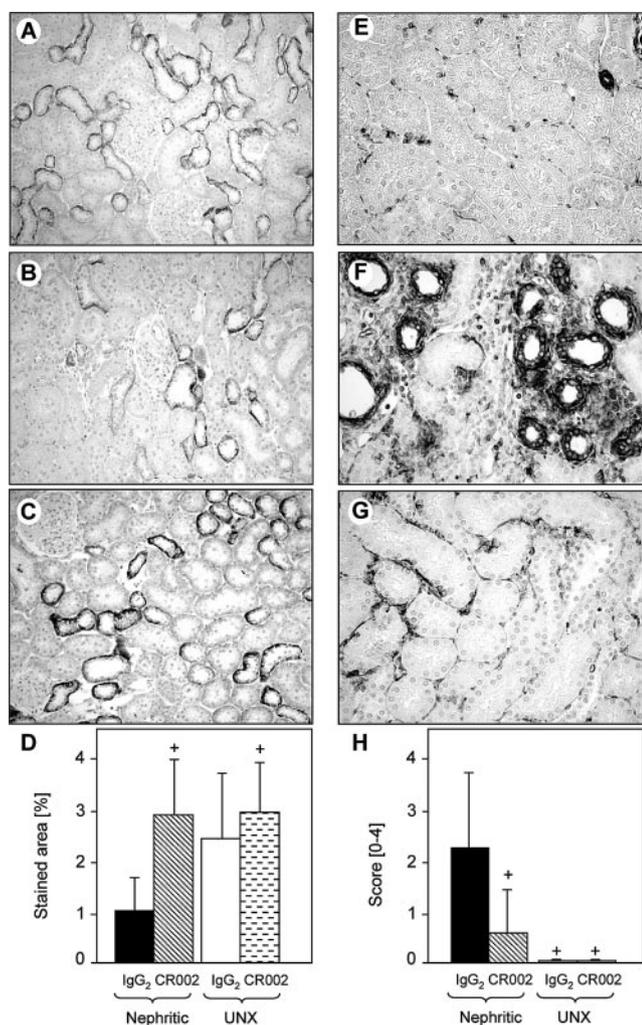
Figure 7. Renal tubular expression of E-cadherin and interstitial expression of vimentin. (A)Section of a nonnephritic, uninephrectomized rat showing widespread tubular expression of E-cadherin. (B) Section of a nephritic rat that received control IgG$_2$ showing E-cadherin expression by a small number of tubular epithelia. (C) Section of a nephritic rat that received CR002 on days 3, 10, and 17, showing widespread tubular expression of E-cadherin, similar to that observed in nonnephritic, uninephrectomized rats. (D) Morphometric evaluation of E-cadherin–positive area in the renal cortex. (E) Section of a nonnephritic, uninephrectomized rat showing vimentin expression mainly in smooth muscle cells of peritubular capillaries and by some interstitial cells. (F) Section of a nephritic rat that received control IgG$_2$ showing markedly increased tubular and tubulointerstitial expression of vimentin. (G) Section of a nephritic rat that received CR002 on days 3, 10, and 17 showing moderate tubulointerstitial vimentin expression. (H) Morphometric evaluation of vimentin-positive area in the renal cortex. Data are means ± SD. [+]$P < 0.05$ *versus* nephritic rats that received control IgG$_2$. Magnifications: ×100 in A through C; ×200 in D through F.

Thy-1.1 antibody clone 1-22-3, Cheng *et al.* (13) already reported that this combination will override the glomerular healing capacity, and both glomerular and subsequently tubulointerstitial scarring occurs. We established that usage of another clone of
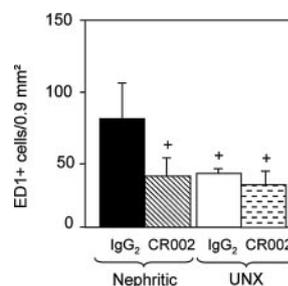
monoclonal Thy-1.1 antibody, OX-7, also results in chronic renal failure. The data of our study confirm that at 8 wk after disease induction, widespread evidence of irreversible renal damage is present in the new model.

After establishing the model, we first demonstrated that in chronic anti–Thy-1.1, analogous to the short-term, reversible model (9), the anti–PDGF-D antibody CR002 acutely reduced mesangial cell proliferation, as evidenced by lower numbers of glomerular mitotic figures and reduced expression of the mesangial cell–specific α-SMA. The major finding of our study was that inhibition of PDGF-D during the acute phase of anti–Thy-1.1 nephritis markedly reduced both glomerular and tubulointerstitial scarring on day 56 after disease induction. PDGF-D inhibition also prevented an increase of serum creatinine on day 49, although the increase in the nephritic IgG control group was not very pronounced. On day 56, the serum creatinine in the nephritic control group was not significantly different from those of all other groups. Albuminuria was not reduced during the course of the disease in the anti–PDGF-D–treated group. Similar effects (a reduction of glomerular and tubulointerstitial scarring in combination with unchanged proteinuria) also has been noted in rats that had puromycin aminonucleoside nephrosis and were treated with inhibitors of complement (28,29) and in rats that underwent 5/6 nephrectomy and were treated with a high dose of angiotensin I–converting enzyme inhibitor (30). Furthermore, in a nearly identical model of mesangioproliferative glomerulonephritis, compared with that used in our study, complement inhibition also reduced tubulointerstitial damage with no change in proteinuria (31). Our results demonstrate that 1 mg/kg body wt CR002 administered at weekly intervals was sufficient to antagonize the biologic activity of PDGF-D *in vivo*. Phase I trials in normal male volunteers are currently under way. One goal of these studies will be to evaluate pharmokinetic properties of CR002 and to do preliminary studies to explore the relationship between CR002 dose and CR002 bound *versus* free PDGF-D in the serum.

The mechanisms by which PDGF-D antagonism prevented chronic renal failure in our model probably include the specific reduction of mesangial expansion during the course of anti–Thy-1.1 nephritis. Mesangial expansion in combination with glomerular hyperfiltration and hypertrophy as a result of previous uninephrectomy (32) will lead to secondary podocyte stretch and damage, a key mechanism in the development of

glomerulosclerosis (33). Podocyte damage indeed was reflected in our study by the widespread *de novo* expression of desmin, a nonspecific marker of podocyte injury (22). CR002 likely acted by reducing glomerular expansion and secondary podocyte damage, as evidenced by lower desmin expression at the glomerular edge. Reduced glomerular damage in nephritic rats that were treated with the PDGF-D antibody may be one of the reasons that subsequent tubulointerstitial damage also was ameliorated. Alternatively, it is conceivable that the persistence of CR002 *in vivo* (Table 1) directly affected the onset of tubulointerstitial fibrosis. At least on a descriptive level, Taneda *et al.* (34) showed that PDGF-D is produced *de novo* in interstitial cells in areas of tubulointerstitial fibrosis in mice with unilateral ureteral obstruction. In follow-up studies, we therefore are addressing the question of whether PDGF-D antagonism also may have a therapeutic role in renal fibrosis in general, *i.e.*, independent of the action on glomerular disease.

Amelioration of tubulointerstitial scarring in nephritic rats that were treated with PDGF-D antibody was associated with a reduction in three key events, which are considered to be of importance in the pathophysiology of renal fibrosis. First, CR002-treated rats exhibited lower tubulointerstitial deposition of the membrane attack complex C5b-9. On the basis of elegant studies in various proteinuric models (23,28,35,36), it now is well established that proteinuria *per se* can induce complement activation in the tubular system, leading to tubular damage and subsequent peritubular inflammation and fibrosis. Second, CR002 prevented the tubular loss of E-cadherin expression and reduced the *de novo* expression of vimentin and α-SMA in renal myofibroblasts. Both of these features are characteristic of EMT, which is viewed as a key event contributing to chronic renal interstitial fibrosis (24–26). Finally, CR002 led to reduced tubulointerstitial monocyte/macrophage influx, which is likely to contribute also to lesser chronic damage. In this regard, it is important to note that PDGF-D was linked recently to monocyte/macrophage infiltration in the skin (37).

Our study is one of the rare examples in which specific growth factor inhibition prevented glomerular and tubulointerstitial scarring. It thereby lends a further basis to clinical trials that aim to intervene in the PDGF system in progressive renal disease. Using a similar model of chronic anti–Thy-1.1 nephritis, we have shown that specific and transient antagonism of PDGF-B also can prevent irreversible renal damage in this model (6). This raises the interesting question of whether (and, if so, how) PDGF-B and -D might interact *in vivo*. Kinetic studies in the acute, reversible anti–Thy-1.1 nephritis model suggested that PDGF-B is an early auto- and paracrine mechanism involved in mesangioproliferative nephritis, whereas PDGF-D seemed to play a role at somewhat later stages and, more important, may also act as an endocrine mechanism (9). The questions of whether PDGF-D represents a "back-up system" for PDGF-B and whether combined inhibition of the two isoforms or, alternatively, blockade of the common PDGF-β receptor might be more effective than antagonism of either growth factor alone remain unresolved. In this respect, it is notable that treatment with Imatinib (STI 571), a nonspecific PDGF receptor tyrosine kinase inhibitor, in acute anti–Thy-1.1 nephritis was no more effective than inhibition of either PDGF-B or -D alone (9,15,38). However, a

comparison of the efficacies of these compounds was never performed in a head-to-head manner.

Conclusion

Our study extends the evidence linking PDGF-D to the pathogenesis of renal damage in mesangioproliferative nephritis and lays the basis for clinical studies to address the question of whether PDGF-D antagonism might represent a novel approach to diseases such as IgA nephropathy or other nephritides that are characterized by augmented mesangial cell proliferation and expansion.

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