The Effects of Platelet-Derived Growth Factor Antagonism in Experimental Glomerulonephritis Are Independent of the Transforming Growth Factor–β System

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Abstract. Platelet-derived growth factor B-chain (PDGF-B)– and transforming growth factor beta (TGF-β)–mediated accumulation of extracellular matrix proteins contributes to many progressive renal diseases. *In vivo,* specific antagonism of either PDGF-B or TGF-β in experimental mesangioproliferative glomerulonephritis resulted in an almost complete inhibition of matrix protein accumulation, which suggests an interaction between signaling pathways of these two growth factors. Because nothing is known on the nature of this possible interaction, PDGF-B was antagonized in the rat anti–Thy 1.1 model of glomerulonephritis by use of specific aptamers and its effects on the TGF-β system were investigated. Antagonism of PDGF-B led to a significant reduction of glomerular matrix accumulation compared with scrambled aptamer-treated nephritic controls. PDGF-B antagonism had no effect on the overexpression of glomerular TGF-β mRNA, TGF-β protein, or the expression of TGF-β receptor type I and II mRNA. By immunohistology, it was possible to detect overexpression of the cytoplasmic TGF-β signaling molecules Smad2 (agonistic) and Smad7 (antagonistic) in glomeruli of nephritic control rats which peaked on day 7 after disease induction, *i.e.,* the peak of mesangial cell proliferation in this model. However, immunohistology and Western blot analysis again revealed no difference in the glomerular expression of both Smad proteins between PDGF-B antagonized and nonantagonized nephritic animals. In addition, no difference in the glomerular expression of phosphorylated Smad2 (P-Smad2) was detected between the differently treated nephritic groups. These observations suggest that the effects of PDGF-B antagonism are independent of TGF-β in mesangioproliferative glomerulonephritides.

Glomerular cell proliferation and accumulation of extracellular matrix contribute to the progressive loss of renal function in many glomerular diseases. Numerous studies both *in vitro* and *in vivo* have implicated platelet-derived growth factor (PDGF) B-chain and transforming growth factor beta (TGF-β) in these pathophysiological processes (1,2).

TGF-β is a multifunctional cytokine that plays a central role in tissue repair, wound healing, and modulation of inflammation (2,3). With respect to matrix synthesis in the kidney, *in vitro* data have demonstrated that TGF-β regulates the production of proteoglycans, fibronectin, and collagens in glomerular mesangial, epithelial, and endothelial cells as well as in tubular cells (4). Several *in vivo* studies have confirmed a profibrotic role of TGF-β in the kidney: glomerulosclerosis and/or glomerular matrix accumulation was induced by overexpression of TGF-β (5,6) or significantly diminished in multiple studies by interfering with the TGF-β system (7–12).

PDGF, in particular the PDGF-B chain, is known to induce glomerular mesangial cell proliferation and extracellular matrix protein accumulation *in vitro* (1,13,14). *In vivo* infusion of recombinant PDGF-B into rats or transfection of glomeruli with PDGF-B cDNA (5,15,16) has resulted in glomerular cell and matrix accumulation. Experiments with a neutralizing antibody against PDGF (17) or, more recently, with a PDGF-B–specific DNA-based aptamer (18), have demonstrated that antagonism of PDGF-B can decrease mesangial cell proliferation and matrix accumulation in the anti–Thy 1.1 mesangioproliferative nephritis in rats. More important, transient antagonism of PDGF-B during the mesangioproliferative phase in the chronic anti–Thy 1.1. model completely prevented the development of renal failure and glomerular as well as tubulointerstitial scaring (19).

In view of the partially overlapping biologic activities of TGF-β and PDGF-B in the glomerulus, the question has arisen whether the production and/or activity of these growth factors is interrelated. Strong evidence for such an interaction is provided by the observation that antagonism of TGF-β or PDGF-B independently resulted in an almost complete inhibition of glomerular matrix accumulation and/or glomeruloscle-

rosis *in vivo* (7,8,18,19). These data also suggest that TGF-β and PDGF-B do not act in parallel but, rather, sequentially *in vivo*. However, the sequence of their activation has been unclear so far, because *in vitro* data obtained with glomerular cells have shown both an induction of TGF-β by exogenous PDGF-B as well as of PDGF-B by exogenous TGF-β (13,20–22). To better understand the relationship between TGF-β and PDGF-B *in vivo,* we specifically antagonized PDGF-B in the rat model of anti–Thy1.1 glomerulonephritis and investigated the effects on the TGF-β system.

Materials and Methods

Aptamer-Based Antagonist against PDGF

The synthesis and characterization of the PDGF-B aptamer (NX1975) have been described in detail elsewhere (18,23). Modifications of the original DNA aptamer (23) 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 (PEG), to prolong its plasma residence time *in vivo* (18). The PDGF-B aptamer bound to rat and human recombinant PDGF-BB with the same affinity ($K_d \sim 0.1$ nM) (18). On the basis of photocrosslinking experiments (23), the aptamer makes a point contact with human PDGF-B-chain at phenylalanine 84 (with isoleucine at position 83).

As a control aptamer, we used a sequence-scrambled analog of the aptamer conjugated to 40 kD PEG (18). The binding affinity of this scrambled aptamer (NX1976) for PDGF-BB ($K_d \sim 1$ μM) is 10,000-fold lower compared with the binding affinity of the PDGF-B aptamer ($K_d \sim 0.1$ nM) (18).

Experimental Model and Experimental Design

All animal studies were approved by the Institutional Review Board. Anti-Thy-1.1 mesangial proliferative glomerulonephritis was induced in 30 male Wistar rats (Charles River, Sulzfeld, Germany), weighing 140 to 160 g at the start of the experiment, by injection of 1 mg/kg monoclonal anti–Thy 1.1 antibody (clone OX-7; European Collection of Animal Cell Cultures, Salisbury, UK). Twenty-four of these rats were treated with the PDGF-B aptamer or the scrambled control dissolved in phosphate-buffered saline (pH 7.4) from days 3 to 7 after disease induction by subcutaneous micro-osmotic pumps (model Alzet 2001; Charles River). The respective daily dose was 5 mg aptamer/kg body weight. The treatment duration was chosen to treat rats from ~1 d after the onset to the peak of mesangial cell proliferation, which in OX-7 induced anti–Thy1.1 nephritis occurs between days 6 and 9 after disease induction. Four groups of rats were studied: (*1*) 12 nephritic rats that received a total of 5 mg each of the PDGF-B aptamer (coupled to 19.6 mg 40 kd PEG), (*2*) 12 nephritic rats that received an equivalent amount of PEG-coupled scrambled aptamer, (*3*) 6 nephritic rats that remained untreated, and (*4*) 6 normal rats. The animals were killed on day 7 after disease induction. A renal cortical section of each rat was obtained for light microscopy. The remaining cortical tissue was pooled within the group with the renal cortex of a second rat in treatment groups 1 and 2. In groups 3 and 4, the renal cortex of three rats each was pooled. This tissue was used to generate a preparation of glomeruli by differential sieving (24). All glomerular isolates were checked microscopically and exhibited a purity >98%. One-half of the isolated glomeruli were used for the preparation of protein lysates and the other half for the isolation of RNA (see below).

For the evaluation of the time course of Smad2 and Smad7 expression in the anti–Thy1.1 model, nephritis was induced in 10 male Wistar rats (Charles River). The animals were killed, and kidney specimens for immunohistology were obtained before disease induction, as well as at 2 h and 7, 10, and 14 d after disease induction ($n = 2$ for each time point).

Renal Morphology

Tissue for light microscopy was fixed in methyl Carnoy's solution and embedded in paraffin. Four-micron sections were stained with the periodic acid–Schiff reagent and counterstained with hematoxylin. In the periodic acid–Schiff–stained sections, the number of mitoses within 30 to 50 glomerular tufts was determined.

Immunoperoxidase Staining

Four-micron sections of methyl Carnoy's–fixed biopsy tissue or formalin-fixed biopsy tissue were processed by an indirect immunoperoxidase technique, as described elsewhere (25). Primary antibodies included an affinity-purified polyclonal goat anti-human/bovine type IV collagen IgG preabsorbed with rat erythrocytes (Biozol, Birmingham, AL), an affinity-purified IgG fraction of a polyclonal rabbit anti-rat fibronectin antibody (Chemicon, Temecula, CA), a polyclonal goat anti-human Smad2 antibody (Santa Cruz Biotechnology, Heidelberg, Germany), a purified polyclonal rabbit anti phospho-Smad2 antibody (raised against the phosphorylated synthetic peptide SS(P)MS(P), kindly provided by P. ten Dijke, Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, the Netherlands), a polyclonal goat anti-human Smad7 IgG (Santa Cruz Biotechnology), and a polyclonal rabbit anti-human TGF-βRII antibody (Santa Cruz Biotechnology). Negative controls consisted of substitution of the primary antibody with equivalent concentrations of normal rabbit or goat IgG. Additional negative controls for the specificity of the Smad2, Smad7, and TGF-βRII immunohistology included overnight preincubation of the specific antibodies with an excess of the appropriate blocking peptides (Santa Cruz Biotechnology). All slides were evaluated by an observer who was unaware of the origin of the slides.

Glomerular staining for type IV collagen and fibronectin was evaluated by use of a semiquantitative scoring system, and the mean score per biopsy was calculated. Each score reflects mainly changes in the extent rather than intensity of glomerular staining and depends on the percentage of the glomerular tuft area showing positive staining: 0, absent staining or <5% of area stained; I, 5% to 25%; II, 25% to 50%; III, 50% to 75%; and IV, >75%.

Immunohistochemical Double Staining

Double immunostaining for the identification of the type of Smad2 or Smad7 expressing glomerular cells was performed by first staining the sections for Smad2 or Smad7 with polyclonal goat anti-human Smad2 or Smad7 antibodies (Santa Cruz Biotechnology) by use of an indirect immunoperoxidase procedure. Sections were then incubated with the IgG$_1$ monoclonal antibody 1A4 against α–smooth-muscle actin. α–smooth-muscle actin in the glomerulus is exclusively expressed by activated mesangial cells. Negative controls included omission of either of the primary antibodies, in which case no double-staining was noted.

Western Blot Analysis

Isolated glomeruli were homogenized in 2 ml of Triton X-100 lysis buffer (50 mM Hepes [pH 7.5], 150 mM NaCl, 1.5 mM MgCl$_2$, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*tetraacetic acid, 10%

glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl chloride, and 0.1 mM sodium orthovanadate) at 4°C. After incubation for 5 min, lysates were centrifuged at 4°C for 15 min at 10,000 × g. The protein concentrations in the lysates were determined according to the method of Lowry *et al.* (26). The soluble lysates were mixed 1:4 with 5× Laemmli buffer and heated for 5 min at 95°C. Soluble lysates (40 μg) were loaded per lane and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis that used 4% acrylamide for the stacking gel and 10% to 15% acrylamide gel for the resolving gels (Smad2-Western, 10%; P-Smad2, 12.5%; and TGF-β and Smad7, 15%). Protein was transferred to nitrocellulose membranes (pore size, 0.45 μm; Schleicher & Schuell, Keene, NH) and probed with polyclonal antibodies against human Smad2 (goat polyclonal antibody, Santa Cruz Biotechnology), phospho-Smad2 (rabbit antiserum raised against the phosphorylated synthetic peptide SS(P)MS(P), kindly provided by P. ten Dijke, Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, the Netherlands), Smad7 (rabbit antiserum [27], kindly provided by C.H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden), and human TGF-β1 (rabbit polyclonal IgG, Santa Cruz Biotechnology). The primary antibodies were detected by use of horseradish peroxidase–conjugated goat anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA) or biotinylated rabbit anti-goat IgG in combination with avidin-coupled horseradish peroxidase (Zymed, San Francisco, CA, and Vector Laboratories Inc., respectively). Negative controls consisted of substitution of the primary antibody with equivalent concentrations of normal rabbit or goat IgG. Because of a presumed up-regulation during glomerulonephritis of some of the antigens that had to be analyzed, protein lysate derived from glomeruli of the scrambled aptamer–treated nephritic control group was used in these controls. Additional negative controls for the specificity of the Smad2 and TGF-β1 Western blotting included overnight preincubation of the specific antibodies with an excess of the appropriate blocking peptides (Santa Cruz Biotechnology). This treatment completely abolished staining of the specific TGF-β1 band and significantly weakened the staining of Smad2 protein.

To investigate whether Smad2 remains phosphorylated during glomerular tissue extraction and processing, two additional nephritic rats were treated with PDGF-B aptamer and the scrambled control. Renal cortical tissue of both animals was lysed and boiled immediately after death. Subsequent anti–phospho-Smad2 Western blot analysis with these tissues gave the same results compared with those of isolated and processed glomeruli, which thereby demonstrates an unaffected phosphorylation during glomerular tissue extraction (data not shown). The antibodies were visualized by the ECL system (Amersham Pharmacia Biotech, Freiburg, Germany). Subsequently, the intensity of the identified bands was quantified by densitometry that used the system of Biostep GmbH (Jahnsdorf, Germany). Specific band intensities were scanned and corrected for the relative intensities of a nonspecific band. The analysis software was from TotalLab (Phoretix International, Newcastle, UK).

Northern Blot Analysis

Total RNA was extracted from the isolated glomeruli with the guanidinium isothiocyanate/phenol/chloroform method by use of standard procedures (28). The RNA content and the purity of the samples obtained was determined by ultraviolet spectrophotometry at 260 and 280 nm. For Northern analysis, the RNA was denatured, and 10 μg/lane were electrophoresed through a denaturing 1% agarose/ formaldehyde gel. The integrity of the RNA was assessed by visualization of ethidium bromide–stained 28S and 18S rRNA bands. Sep-

arated RNA was then transferred onto a nylon membrane (Hybond N, Amersham Pharmacia Biotech) by capillary blotting and cross linked with the use of ultraviolet light at 310 nm. Radioactive α-^{32}P–labeled cDNA probes were generated by use of the Megaprime DNA labeling system (Amersham Pharmacia Biotech), and hybridization was performed with the QuickHyb hybridization solution (Stratagene, Heidelberg, Germany). Band intensities were scanned as described above and corrected for the relative intensities of the 28S rRNA signal on the membrane.

For the detection of TGF-βRI and TGF-βRII mRNA, the hybridization probes were created by reverse transcription–PCR amplification that used the following gene-specific primers: rat TGF-βRI (680-bp fragment) forward, 5'-CGTCTGCATTGCACTTATGC-3' and reverse, 5'-CTGTGGCAGAATCATGTCTC-3'; and rat TGF-βRII (650-bp fragment) forward, 5'-CACTGTCCACTTGTGA-CAAC-3' and reverse, 5'-GGTCTCAAACTGCTCTGAAG-3'. The mouse TGF-β1 probe used encoded part of the mature protein according to Genbank accession number M13177, nt 1220 to 1522 (29). The Smad2-specific hybridization probe was generated by restriction digestion of an expression construct for Flag tagged Smad2, which was described in Nakao *et al.* [30].

Reverse transcription–PCR reactions with the above-listed oligonucleotides were performed in a 30-μl reaction mix that included 4 μg of total rat liver RNA, 1 μl of oligo-dT primer (12 to 18 nt, 0.5 μg/μl, Life Technologies/BRL), 6 μl of M-MLV reverse transcriptase buffer (Life Technologies/BRL), 1.5 μl dNTP mix (10 mM each, Amersham Pharmacia Biotech), 1 μl RNase inhibitor (40 U/μl, Roche), 2 μl of M-MLV reverse transcriptase (200 U/μl, Life Technologies/BRL), and diethyl pyrocarbonate–treated H$_2$O. The mix was incubated for 1 h at 42°C, 5 min at 80°C, and 1 min on ice and subsequently centrifuged; 1 μl of the cDNA was included in a standard 50-μl polymerase reaction mix and amplified under standard conditions with the appropriate primers. The amplified products were separated on a 1.5% agarose gel, eluted from the gel with the QIAEX method (Quiagen, Hilden, Germany), and used as probes in the Northern blots.

Statistical Analyses

All values of the PDGF-B– and scrambled aptamer–treated animals are expressed as mean ± SD. Statistical significance (defined as $P < 0.05$) was evaluated by ANOVA and Bonferroni t tests. The values for the untreated nephritic and normal animals are expressed as the mean and are excluded from the statistical analysis because only two samples per group (representing pooled tissue from three rats) were analyzed.

Results

Effect of PDGF-B Antagonism in Anti–Thy1.1 Nephritis on Glomerular Extracellular Matrix Protein Expression and Glomerular Cell Proliferation

We first confirmed that antagonism of PDGF-B by aptamers reduced glomerular matrix accumulation and cell proliferation similar to prior results *in vivo* (18,19). In renal biopsies obtained from nephritic rats on day 7 after disease induction, treatment with the PDGF-B aptamer indeed significantly reduced mesangioproliferative changes. Thus, the glomerular accumulation of fibronectin and type IV collagen in PDGF-B aptamer–treated rats was almost normalized and was significantly reduced in comparison to rats that received scrambled aptamer (Figure 1). Additionally, the PDGF-B aptamer signif-
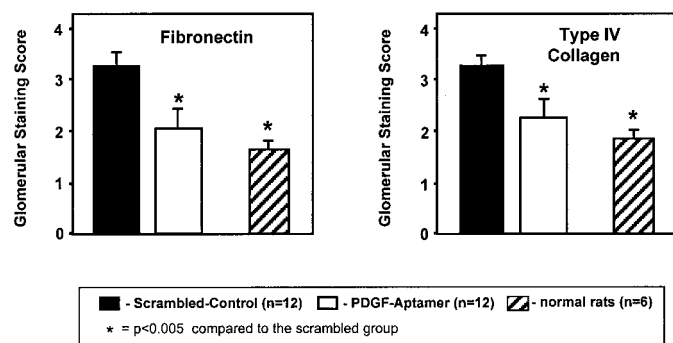
Figure 1. Glomerular extracellular matrix protein staining on day 7 after disease induction in rats that received either the platelet-derived growth factor (PDGF) aptamer or the scrambled aptamer. Glomerular expression of the respective protein in six normal rats is included. *P < 0.005.

icantly reduced the number of glomerular mitoses in comparison to the scrambled control (2.7 ± 1.9 *versus* 9.7 ± 3.9 mitoses per 100 glomerular cross-section, respectively; P < 0.005).

Effect of PDGF-B Antagonism in Nephritic Rats on the Glomerular Expression of TGF-β1 and its Receptors

As shown in Figure 2A, glomerular TGF-β1 mRNA was up-regulated on day 7 after induction of the anti–Thy1.1 nephritis. Up-regulation of TGF-β1 mRNA and protein in this model is consistent with reports elsewhere (7,9,31–34). More important, treatment of nephritic rats with the PDGF-B aptamer from day 3 to 7 had no effect on the glomerular expression of TGF-β1 mRNA on day 7 compared with the scrambled aptamer–treated group and the untreated nephritic control group. Two glomerular TGF-β1 transcripts of 2.5 and 1.9 kb size were detected with the 2.5-kb mRNA as the predominant form. Similarly, glomerular TGF-β1 protein expression was up-regulated on day 7 and did not differ between PDGF-B aptamer–treated animals and the nephritic control groups (Figure 2B). Strong binding forces between two TGF-β1 monomers probably resisted the normal denaturing Western blot conditions and led to the detection of the active dimer form (Figure 2B). This phenomenon has also been demonstrated elsewhere (35).

Recently, glomerular up-regulation of TGF-β receptors I and II has been demonstrated in rats with anti–Thy1.1 nephritis (36), which paralleled the overexpression of TGF-β in this model. To investigate a potential effect of PDGF-B antagonism in glomerulonephritis on glomerular TGF-β receptor expression, Northern blotting was performed for TGF-β receptor I and II. As shown in Figure 2C, receptor mRNA was up-regulated in the nephritic state. However, treatment with the PDGF-B aptamer did not result in any significant changes of TGF-β receptor I or II transcript expression on day 7 after disease induction compared with the nephritic control groups. Furthermore, glomerular TGF-β receptor II immunostaining demonstrated an up-regulation of receptor protein in all nephritic groups compared with normal animals. Protein expres-

sion was largely confined to nodules of proliferating mesangial cells. Again, we did not detect differences between the nephritic treatment groups (data not shown).

### Smad2 and Smad7 Expression in the Anti–Thy1.1 Nephritis

Smad2 and Smad7 are central mediators in the TGF-β signaling system (30,37,38). Therefore, an analysis of the expression and activation of these proteins provides insight into the TGF-β induced cellular activation. Because little is known about the glomerular localization of Smad2 and Smad7 in the anti–Thy1.1 nephritis model, we first evaluated the time course of their expression. In kidneys of healthy controls, occasional positive Smad2 staining was noted in the peritubular and glomerular capillary endothelium (Figure 3A). Renal arteriolar endothelium also stained positively. This expression pattern did not change during the course of the disease, but a significant increase in Smad2 expression was noted on day 7 in glomerular capillaries (Figure 3B) that decreased to the level of the control animals on days 10 and 14. Smad2/α–smooth-muscle actin double immunostaining in the renal tissues confirmed a predominant nonmesangial expression of Smad2 (data not shown). Smad7 expression was absent in the glomerulus of healthy controls but was present in arterioles and some distal tubular and/or collecting duct cells (Figure 4A). Widespread glomerular Smad7 expression in a mesangial pattern first appeared at 2 h after disease induction, reached its maximum on day 7 (Figure 4B), and decreased thereafter. In parallel, tubular Smad7 expression also transiently became more widespread (Figure 4B). A predominant mesangial expression of Smad7 on day 7 after disease induction was confirmed by Smad7/α–smooth-muscle actin double immunostaining (data not shown). Despite evidence of Smad2 and Smad7 regulation at the transcriptional level (39,40), at least in the case of Smad2 we did not detect glomerular overexpression on day 7 in nephritic rats (data not shown).

Effect of PDGF-B Antagonism in Nephritic Rats on the Glomerular Expression of Smad2 and Smad7

Treatment of the nephritic rats with the PDGF-B antagonist did not change the glomerular Smad2 overexpression on day 7 after induction of the anti–Thy 1.1 nephritis (Figure 3C), and the staining pattern and intensity were indistinguishable from those observed in nephritic rats that received the scrambled control aptamer (data not shown). Additionally, there was no detectable difference between the Smad2 staining pattern in PDGF-B aptamer–treated animals (Figure 3C) and the expression of this protein in untreated nephritic animals on day 7 (Figure 3B). Similarly, glomerular Smad7 overexpression was not different between untreated (Figure 4B), PDGF-B aptamer–treated (Figure 4C) or scrambled aptamer–treated (data not shown) nephritic rats on day 7 after disease induction. Similar to the data obtained by immunohistochemistry, Western blot analysis of glomerular Smad2 (Figure 6, upper blot) and Smad7 (Figure 5) expression also did not detect significant
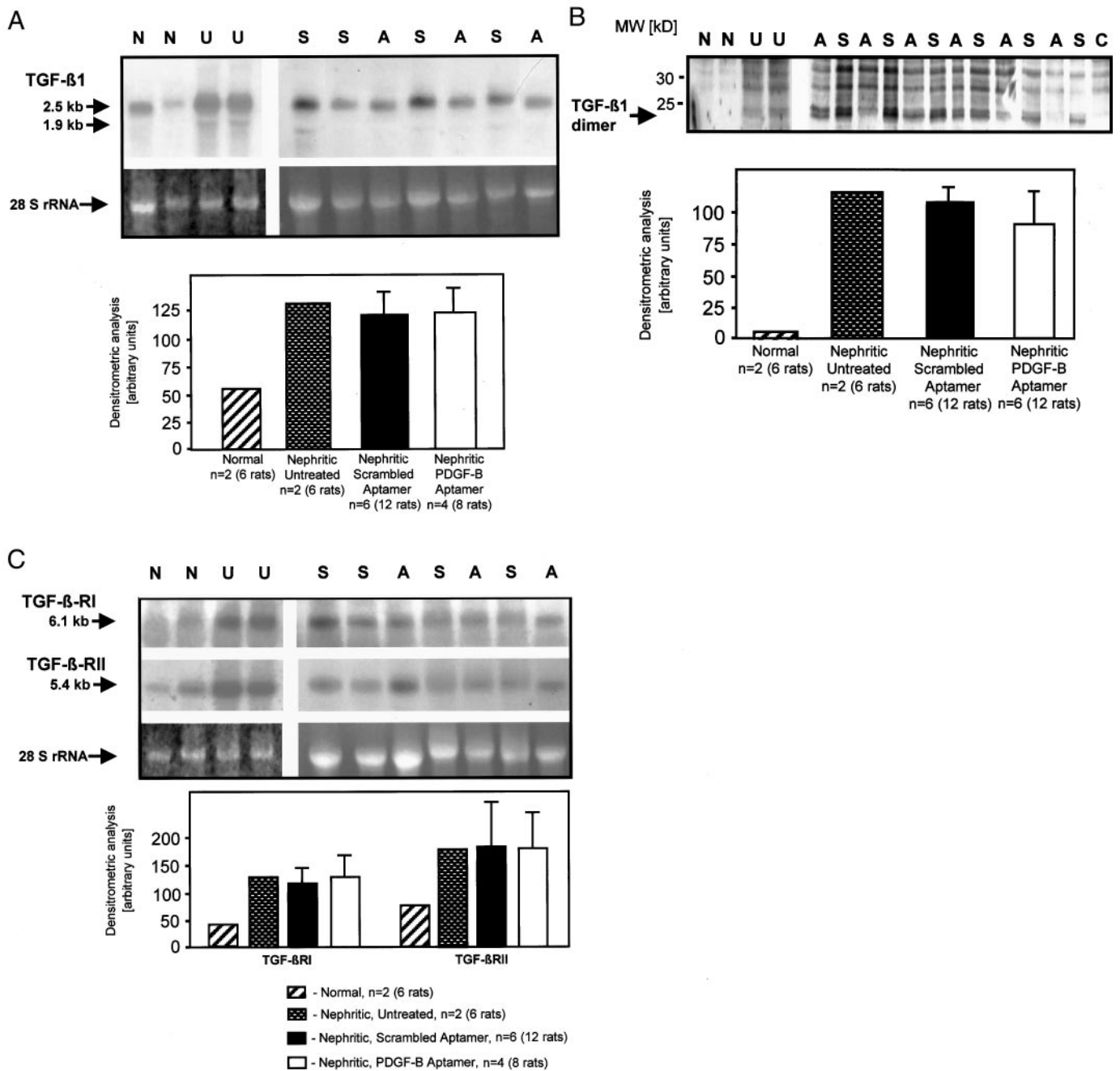
Figure 2. Glomerular expression of (A) transforming growth factor beta (TGF-β1) transcripts, (B) TGF-β protein, and (C) TGF-β receptor I and II transcripts on day 7 after induction of anti–Thy 1.1 nephritis in normal rats, untreated nephritic rats, or nephritic rats that received either the PDGF aptamer or the scrambled aptamer. (A) and (C) Northern blot analysis combined with subsequent densitometry; each membrane slot represents pooled glomerular RNA of three animals ("Normal" and "Nephritic, untreated" groups) or of two animals (aptamer-treated groups). Four representative samples of scrambled aptamer–treated rats and three representative samples of PDGF aptamer–treated rats are shown. In the PDGF aptamer–treated group, two samples were degraded due to RNase contamination, which explains the lower n of 4 in this group in the densitometric analysis. Band intensities were corrected for the relative intensities of the 28S rRNA signal on the membrane. (B) Western blot analysis combined with subsequent densitometry; each slot represents pooled glomerular protein lysates of three animals ("Normal" and "Nephritic, untreated" groups) or of two animals (aptamer-treated groups). The experiment was repeated twice with similar results. n, normal group; U, untreated nephritic group; A, nephritic PDGF aptamer–treated group; S, nephritic scrambled aptamer–treated group; and C, negative control.
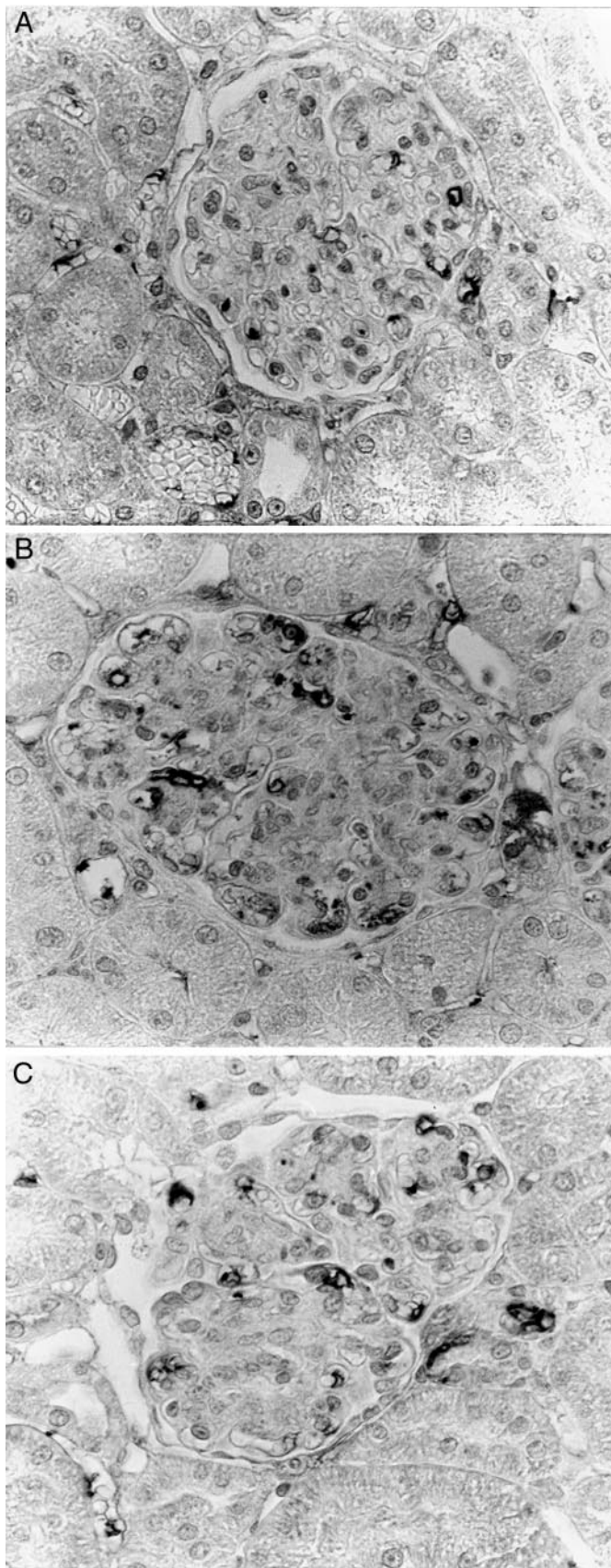
Figure 3. Immunohistological detection of Smad2 in normal, untreated nephritic, and PDGF aptamer–treated nephritic rats. Renal expression of Smad2 in a normal rat is confined to the peritubular and

differences between PDGF-B aptamer–treated rats and nephritic control rats on day 7 after disease induction.

### *Effect of PDGF-B Antagonism in Nephritic Rats on Smad2 Activation*

Our failure to detect differences in the glomerular overexpression of the agonistic TGF-β signaling protein Smad2 between PDGF-B aptamer–treated and scrambled aptamer–treated nephritic rats does not exclude the possibility that there may be differences in the degree of Smad2 activation. Upon ligand binding, Smad2 interacts with the TGF-β receptor I chain, becomes phosphorylated, and translocates in a heteromeric complex of Smad2, Smad3, and Smad4 to the nucleus (30). We therefore also investigated the expression of the activated, *i.e.,* phosphorylated, Smad2 protein (P-Smad2) in nephritic rats that received either the PDGF-B or scrambled aptamer. As demonstrated by Western blot analysis (bottom panel of Figure 6) and densitometry, we again did not detect a significant difference in the activation of Smad2 between PDGF-B aptamer–treated animals and the nephritic controls. Finally, P-Smad2 immunohistology in renal tissue demonstrated nuclear localization of the activated Smad2 (Figure 7). In normal rat tissue, P-Smad2 staining predominantly localized to nuclei in the glomerular endothelium, occasional parietal glomerular epithelial cells, and endothelial and smooth-muscle cells of small arterial vessels as well as peritubular endothelial cells (Figure 7). Although we cannot formally exclude that occasional mesangial nuclei also contained P-Smad2 in normal rats, the expression pattern of P-Smad2 appeared to largely correspond to that of Smad2 (Figure 3). In nephritic animals, the overall localization of nuclear P-Smad2 expression did not change, but more glomerular nuclei showed positive staining. Again, we did not detect a difference in the expression of P-Smad2 between PDGF-B aptamer–treated nephritic animals and the nephritic controls.

Discussion

In this study, we first established that, under our experimental conditions, the PDGF-B aptamer was as potent in reducing glomerular extracellular matrix protein accumulation and mesangioproliferative changes as it was in our previous studies (18,19), in which two different anti–Thy1.1 nephritis models had been investigated (a reversible and a progressive model). In this study, the aptamer led to reductions of mesangioproliferative changes and glomerular accumulation of fibronectin and type IV collagen comparable to those observed elsewhere. Because it has been well established that the acute phase in the anti–Thy1.1 nephritis model is dependent on PDGF (17–19),

glomerular capillary endothelium as well as to renal arteriolar endothelial cells Panel A. (B) In anti–Thy 1.1 nephritis, the number of Smad2-expressing renal capillary endothelial cells is increased on day 7 after disease induction. (C) Immunohistological detection of Smad2 in PDGF aptamer treated rats on day 7 of the nephritis revealed no differences in the expression pattern or intensity to the respective staining in untreated nephritic rats on day 7 (B). Magnification, ×600.
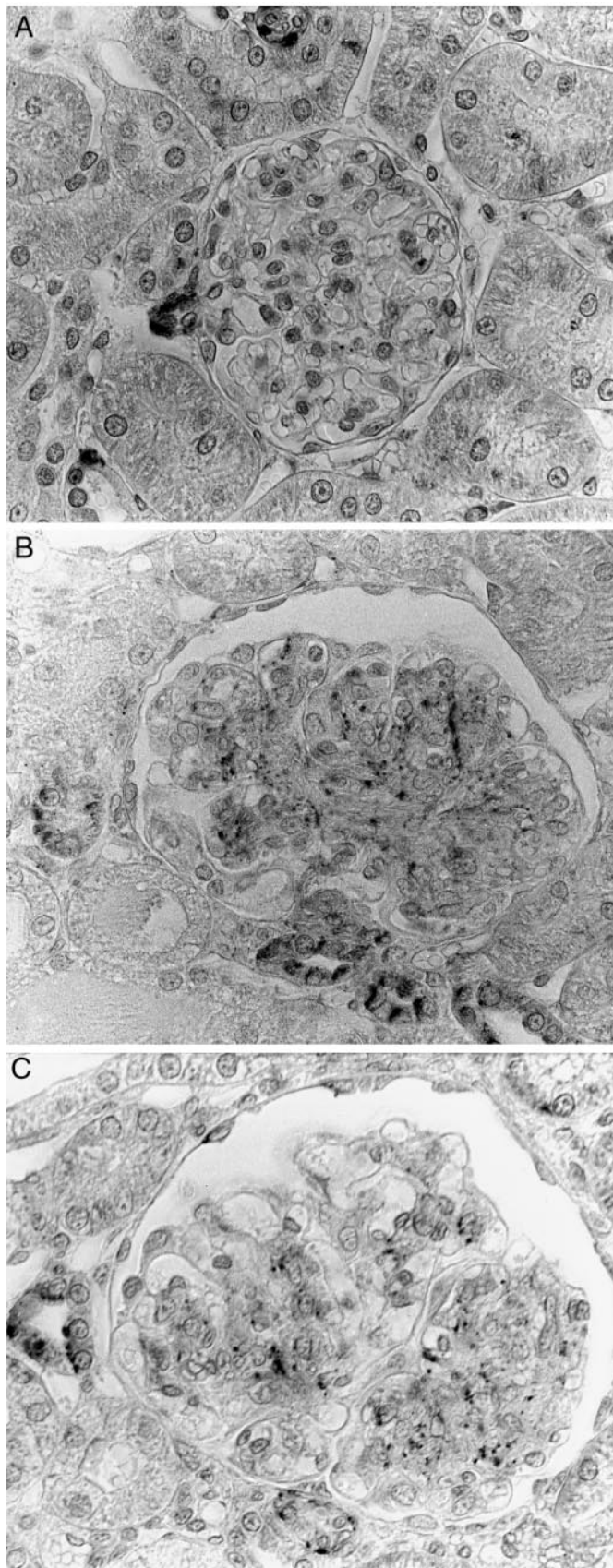
Figure 4. Immunohistological detection of Smad7 in normal, untreated nephritic, and PDGF aptamer–treated nephritic rats. (A) Renal expression of Smad7 in a normal rat is confined to some distal tubular
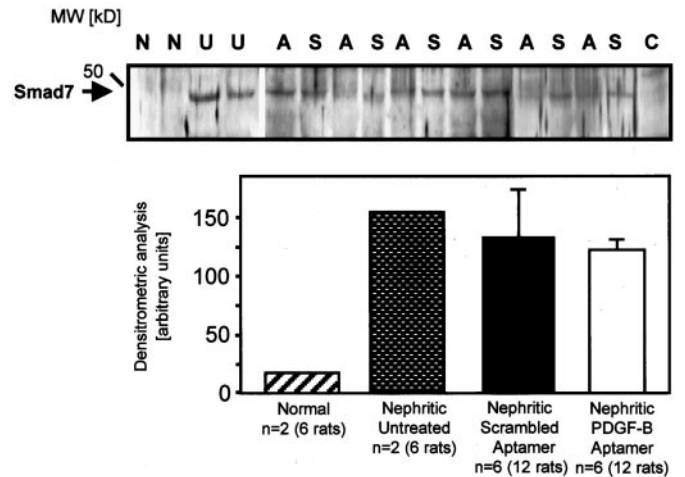
Figure 5. Glomerular expression of Smad7 protein. Western blot analysis combined with subsequent densitometry; each slot represents pooled glomerular protein lysates of three animals ("Normal" and "Nephritic, untreated" groups) or of two animals (aptamer-treated groups). The experiment was repeated twice with similar results. MW, molecular weight.

these data, together with our recent *in vitro* results (18), substantiate the notion that the aptamer acted *in vivo* by specifically antagonizing PDGF-B chain.

Next, we assessed the glomerular expression of components of the TGF-β system. Elsewhere, by use of an anti–Thy1.1 model induced by injection of anti-thymocyte serum into rats, glomerular overexpression of TGF-β as well as its receptors, TGF-βRI, -βRII, and -βRIII, has been demonstrated to coincide temporally with the peak of mesangial cell proliferation (8,35,40). In this study, we therefore confined our analyses to day 7, *i.e.,* the time point at which maximal mesangial expansion occurs in the anti–Thy 1.1 glomerulonephritis induced by monoclonal antibody OX-7. Because the regulation of the biologic activity of TGF-β is extremely complex (41), we also attempted to obtain more direct evidence for TGF-β activity *in vivo* by analyzing intracellular molecules that are induced upon TGF-β signaling, namely the members of the Smad family. We could demonstrate a constitutive renal expression of Smad7 and, for the first time, of Smad2 and P-Smad2, which was augmented during mesangioproliferative nephritis and again peaked around day 7 in the anti–Thy 1.1 glomerulonephritis. In contrast to the predominant glomerular endothelial expression of Smad2 during glomerulonephritis, glomerular expresssion of Smad7 in the nephritic state was mainly located to the

cells. (B) Maximum Smad7 expression is noted on day 7 of anti–Thy1.1 nephritis with apparent *de novo* glomerular expression and an increased number of positively stained tubular cells. (C) Immunohistological detection of Smad7 in PDGF aptamer–treated rats on day 7 of the nephritis revealed no differences in the expression pattern or intensity to the respective staining in untreated nephritic rats on day 7 (B). Magnification, ×600.
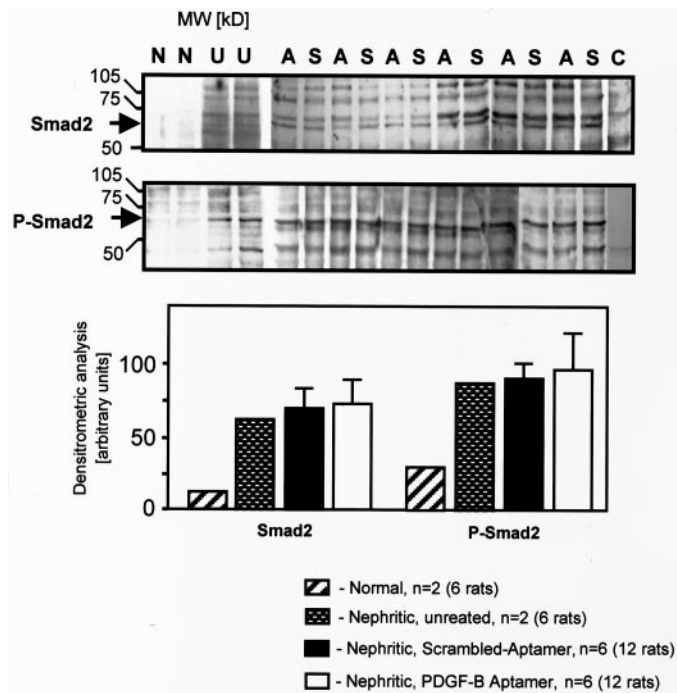
Figure 6. Glomerular expression of Smad2 protein and detection of activated phosphorylated Smad2. Western blot analysis combined with subsequent densitometry; each slot represents pooled glomerular protein lysates of three animals ("Normal" and "Nephritic, untreated" groups) or of two animals (aptamer-treated groups). The experiment was repeated twice with similar results.
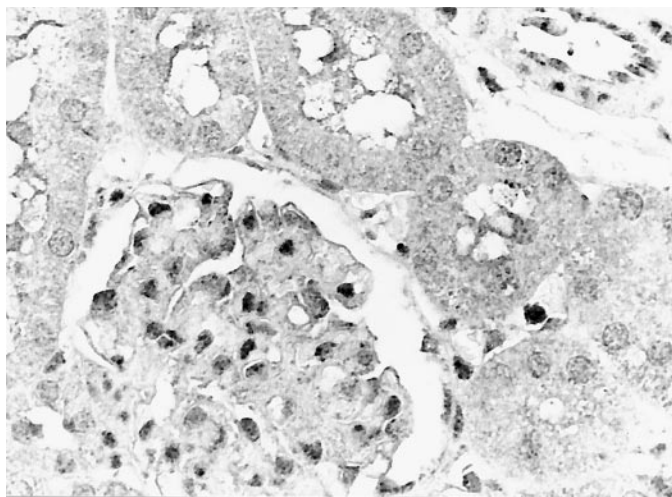

Figure 7. Immunohistological detection of phosphorylated Smad2 in rat renal tissue. Renal expression of P-Smad2 in a normal rat is mainly confined to nuclei of the glomerular capillary endothelium, occasional parietal epithelial cells, nuclei of arteriolar endothelial and smooth-muscle cells, and peritubular endothelial cells. Magnification, ×600.

mesangium. Very recently, constitutive renal Smad7 expression has also been demonstrated by Uchida *et al.* (34), who, in contrast to our study, noted no up-regulation of its expression during experimental mesangioproliferative glomerulonephritis. The apparent difference between the data of Uchida *et al.* and

our observations likely results from the different animal model used in this study and different staining techniques. Instead of specific nuclear staining during immunohistology with the anti-Smad2 antibody, we mainly noted cytoplasmic and sometimes overall cellular staining. This phenomenon is similar to c-myc oncoprotein, another nuclear factor, and might depend on tissue fixation conditions (42). However, specific nuclear staining of activated Smad2 was noted with the anti–P-Smad2 antibody. The constitutive activation of Smad2 in some cells in normal kidneys is consistent with the low expression level of TGF-β1 and its receptors in normal individuals. Apart from TGF-β, the only other known inducer of Smad2 is activin (43). Other inducers of Smad7 are activin and bone morphogenetic protein, all members of the TGF-β superfamily (43). Very recently, it has been described that activin, like TGF-β, is overexpressed in the mesangium during the course of anti–Thy1.1 nephritis (44). Thus, our demonstration that Smad2 and Smad7 are overexpressed in anti–Thy1.1 nephritis may result from both TGF-β and activin signaling. With this limitation in mind, we assessed the effects of PDGF-B antagonism on the various components of the TGF-β system.

The central finding of this study was that highly specific antagonism of PDGF-B in experimental mesangioproliferative nephritis did not affect the glomerular expression of TGF-β1 mRNA and protein, the mRNA of its receptors TGF-βRI and TGF-βRII, and TGF-βRII protein. In addition, the glomerular overexpression of Smad2 and Smad7, and, more important, phospho-Smad2, was also not affected by PDGF-B antagonism. Although, as noted above, the overexpression of Smad2 and Smad7 is not absolutely specific for TGF-β activity *in vivo,* the lack of an effect exerted by the PDGF-B aptamer strongly suggests that TGF-β downstream signaling was not affected by the PDGF antagonism.

Currently available data have implied that in the anti–Thy1.1 model, TGF-β and PDGF-B act serially in the evolution of mesangioproliferative changes. Our complete failure to demonstrate effects of PDGF-B antagonism on components of the TGF-β system leads to the conclusion that PDGF-B does not act upstream but rather downstream of TGF-β under the conditions of this study. However, because we cannot formally exclude the possibility that PDGF-B and TGF-β might even act independently of each other *in vivo,* the final evidence for this conclusion should come from *in vivo* TGF-β antagonism studies in glomerulonephritis and the analysis of the PDGF system under these conditions. What is the potential therapeutic implication of the present findings? Specific antagonism of PDGF-B in adult life apparently is safe. Thus, mice transgenic for a circulating PDGF antagonist (the extracellular domain of the PDGF β-receptor), which was produced starting in late embryogenesis and adulthood only, showed no phenotypic abnormalities (45). Furthermore, both experimental renal studies (46), as well as phase I and II studies with orally available PDGF-B antagonists in tumor patients, have shown little toxicity (47). In the case of TGF-β, no such data are presently available. Rather, it has been shown that TGF-β, in addition to its profibrotic role, has strong immunosuppressive activity and that mice deficient in TGF-β die of a multifocal inflammatory

disease as soon as maternal transfer of TGF-β ceases (48). Furthermore, in contrast to the established role of TGF-β in mediating glomerular matrix accumulation, its relevance for the regulation of mesangial cell proliferation is less clear. At least *in vitro*, TGF-β can act as a mesangial mitogen at low concentrations but inhibits mesangial cell growth at higher levels (49) and might thereby even limit pathologic mesangial cell proliferation *in vivo*. Finally, in contrast to antagonism of PDGF-B, no study so far has demonstrated that inhibition of TGF-β in the anti–Thy1.1 model can provide a long-term benefit that will extend beyond the short-term inhibition of matrix accumulation. Given these considerations, antagonism of PDGF-B appears to represent a safe therapeutic approach, because it does not affect the very complex TGF-β system and the rationale for inhibiting PDGF-B in mesangioproliferative disease is strengthened.

Acknowledgments

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