Pentoxifylline Attenuated the Renal Disease Progression in Rats with Remnant Kidney

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Abstract. Previous studies have reported that pentoxifylline, a phosphodiesterase inhibitor, attenuates experimental mesangial proliferative glomerulonephritis. This study hypothesized that pentoxifylline could also attenuate the renal disease progression in rats with remnant kidney. After 5/6 subtotal nephrectomy, rats developed progressively elevated proteinuria and plasma creatinine, glomerulosclerosis, interstitial inflammation, and fibrosis, all of which were attenuated by 40 to 60% by pentoxifylline. However, the elevated BP was not changed by pentoxifylline. Pentoxifylline reduced the upregulation of monocyte chemoattractant protein-1 gene by 60% in the cortex of remnant kidney. Furthermore, pentoxifylline was found to decrease the numbers of interstitial myofibroblasts by 60% in the cortex of remnant kidney and suppress the proliferation of connective tissue growth factor gene in cultured fibroblasts and mesangial cells. Combining pentoxifylline with an angiotensin-converting enzyme inhibitor, cilazapril, almost completely attenuated the renal disease progression in rats with remnant kidney. In conclusion, pentoxifylline alone can attenuate the chronic renal disease progression. Its combination with cilazapril has the potential to prevent the renal disease progression almost completely.

Almost all forms of kidney diseases progressing to end-stage renal failure are characterized by diffuse fibrosis (1). Whatever the initial injury, the remaining nephrons undergo adaptive hypertrophy and hyperfiltration that minimize the functional consequences of the progressive nephron loss (2,3). However, the adaptation ultimately leads to a vicious cycle in which hyperfiltration of the remaining nephrons impairs glomerular barrier, which in turn induces tubulointerstitial damage and then results in the loss of more nephrons (4,5). Both angiotensin-converting enzyme inhibitor (ACEI) and angiotensin II receptor blocker (ARB) are shown to break the vicious cycle and protect kidneys from progressive injury. However, the ultimate step of halting renal disease progression in the long term is not achieved with either drug alone, especially when the treatment is started during the late course of the renal disease (6–8). Furthermore, hyperkalemia frequently complicates the treatment (9). In addition to ACEI and ARB with the well-recognized renoprotective effect, mycophenolate mofetil, a specific anti-lymphocyte agent, has been shown to attenuate the progression of remnant kidney, suggesting a novel therapy for chronic renal disease through reducing the infiltration of inflammatory mononuclear cells (8,10,11).

Pentoxifylline (PTX) is a phosphodiesterase inhibitor used clinically to treat patients with peripheral vascular diseases (12). In addition to its hemorheologic activity, many clinical studies have shown that PTX decreases the proteinuria of patients with diabetic or membranous nephropathy and improves the disease of patients with multiple sclerosis or human T lymphotropic virus type I-associated myelopathy (13–17). PTX also inhibits the proliferation of cultured lymphocytes, fibroblasts, and mesangial cells, and it reduces the production of extracellular matrix proteins (16,18–20). Furthermore, PTX can attenuate experimental cyclosporine nephropathy and mesangial proliferative glomerulonephritis (21,22).

Thus, we hypothesized that PTX could attenuate the progression of chronic renal disease through its suppression on inflammation, cell proliferation, and fibrosis. To test this hypothesis, we first used the rat model of 5/6 subtotal nephrectomy to study the in vivo effects of PTX on renal function, pathology, and the expression of proinflammatory and profibrogenic genes. Second, we used in vitro cultures of proximal tubular cells, mesangial cells, and interstitial fibroblasts to study the possible mechanisms for the renoprotective effect of PTX. Finally, we compared the effects of combining PTX with...
an ACEI, cilazapril (CLZ), versus either drug alone on the renal disease progression in this rat model.

**Materials and Methods**

**Animals and Surgery**

Male Wistar rats weighing 220 to 260 g were obtained from the Experimental Animal Center in our institute. All rats were housed in a constant-temperature room with a consistent light cycle (light from 07:00 to 18:00) and fed a standard rat chow (protein 23.4%). The animal care and treatment were conducted according to the guidelines of the National Science Council of the Republic of China (NSC 1997). On the day of the operation, all rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally). Renal mass reduction (RMR) was obtained by right nephrectomy and ligation of two or three branches of the left renal artery according to the method modified from the report of Floege et al. (3). Sham operation (SHM) was performed by a laparotomy and manipulation of renal pedicle only in age-matched rats used as controls.

**Experimental Design**

Daily water intake averaged 30 ml during the first 2 wk after surgery. This intake amount was used to estimate the dosage of drug administration. The healing of the surgical wound was poor in rats receiving PTX (Sigma, St. Louis, MO) immediately from the day of the surgery in pilot study. The body weight was also much lower. The accumulated mortality rate was 37.5% in PTX-treated RMR rats; however, there was no mortality in vehicle-treated RMR rats during an 8-wk observation period (n = 8 for each group). We therefore started PTX treatment from day 7 after surgery, when the surgical wound was healed. Thereafter, there was no more mortality in rats receiving PTX treatment. The first set of animal experiments was designed to study the effect of PTX on the renal disease progression in rats with remnant kidney. RMR rats were randomly divided into two groups receiving either PTX (400 mg/L in the drinking water; n = 16), or vehicle (drinking water only, n = 16) from day 7 to week 8 after surgery. Thirty-two SHM rats were studied in parallel. Body weight was recorded, and 24-h urine samples were collected in metabolic cages on days 0 and 7 and thereafter expressed as the percentage of glomeruli per g renal mass, and divided into three parts. One was fixed in 10% neutral buffered formalin for histochemistry and immunohistochemistry, another was fixed in 4% paraformaldehyde for total RNA extraction, and the other was dissected to isolate the cortex, which was quickly frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

The second set of animal experiments was designed to compare the effect of combining PTX with CLZ (Roche, Basel, Switzerland) versus either drug alone on the progressive renal disease in RMR rats. Forty-eight RMR rats were divided into four groups receiving either vehicle (drinking water only, n = 12), PTX (400 mg/L in the drinking water; 45 to 55 mg/kg body wt per day; n = 12), CLZ (25 mg/L in the drinking water, 3 to 3.5 mg/kg body wt per day; n = 12), or PTX + CLZ (n = 12) from day 7 to week 8 after surgery. Eight age-matched SHM rats served as normal controls. After collecting the 24-h urine samples in metabolic cage on week 8, all rats were anesthetized to measure the MABP. Finally, the fragments of remnant kidneys were taken for pathology and total RNA extraction.

**Renal Pathology**

The specimens were immersed in 30% sucrose/PBS at 4°C overnight after fixation in 4% paraformaldehyde/PBS at 4°C. The specimens were then embedded in Tissue-Tek OCT compound (Miles Inc., Elk hart, IN) in isopentane in liquid nitrogen and stored at −70°C until cryostat sectioning. Mouse monoclonal antibodies were used for the immunohistochemical detection of the following antigens: (1) ED1 antigen present in rat monocytes and macrophages (Chemicon, Temecula, CA); (2) a monomorphic determinant of the rat I-A antigen, MHC class II antigen, present on B lymphocytes, dendritic cells, and macrophages (OX-6 clone) (Serotec, Oxford, United Kingdom); (3) rat CD4 cell surface glycoprotein, a 55-kd molecule expressed by helper T cells, thymocytes, and macrophages (W3/25 clone) (Serotec); (4) rat CD8 cell surface glycoprotein expressed by a subset of T lymphocytes, most thymocytes, and most NK cells (OX-8 clone) (Serotec); (5) α-smooth muscle actin (α-SMA) present in myofibroblast (1A4 clone; Sigma) Briefly, 5-μm renal sections were micro waved (Tatung TMO-6810) in 0.01 M citrate buffer (pH 6.0) for 5 min at 800 W (23). They were allowed to cool for 15 min, rinsed in distilled water twice and in PBS for 5 min. The sections were then treated with 0.5% hydrogen peroxide/PBS for 20 min at room temperature to block the endogenous peroxidase. They were subsequently blocked with 10% normal goat serum for 30 min at room temperature, and then incubated with primary antibodies at 4°C overnight. The sections were washed three times in PBS/0.2% Triton X-100 for 10 min and incubated with biotin-conjugated secondary antibodies (DAKO, Carpenteria, CA) for 1 h at room temperature the following day. They were then incubated with the avidin-biotin peroxidase reagent (DAKO) for another 1 h at room temperature. After three washes in PBS/0.2% Triton X-100 for 10 min each, the
reactions on sections were detected with peroxidase substrate containing dianminobenzidine chromagen (DAKO). The specific antibodies were omitted in sections as negative controls. The cells positive for specific antigen were counted in 20 consecutive high-power microscopic fields (×400) of the cortical interstitium using a 0.0625-mm² ocular grid for each rat. This examination avoided glomeruli and large vessels. Data from each group were expressed as mean ± SD per 0.0625 mm². The observer was blinded to the animal group.

**Rat Mesangial Cell Culture**

Rat mesangial cells were cultured and identified as stated previously in the Materials and Methods (18). Mesangial cells of passages 4 to 10 were used for experiments and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 5 μg/ml insulin at 37°C in a 5% CO₂ humidified incubator. Confluent cells were made quiescent by placing them in RPMI 1640 medium supplemented with 0.5% FCS and 5 μg/ml insulin for 24 h. They were then stimulated with 1 μM angiotensin II (AngII, Sigma) or 5 ng/ml transforming growth factor-β1 (TGF-β1; R&D Systems, Minneapolis, MN) in the presence or absence of PTX.

**Rat Proximal Tubular Cell Culture**

Rat proximal tubular cells were cultured and identified as stated previously in the Materials and Methods (24). In brief, proximal tubular cells were isolated from renal cortex in 45% Percoll/Krebs-Henseleit-saline buffer (Sigma) by centrifugation after collagenase digestion. The band enriched in proximal tubular cells was withdrawn, resuspended in DMEM/F12 medium supplemented with 10% FCS and 5 μg/ml insulin, and cultured in 60-mm petri dishes overnight at 37°C, 5% CO₂ in a humidified incubator. The culture medium was then changed to DMEM/F12 medium supplemented with 5 μg/ml insulin and 10 ng/ml epidermal growth factor (R&D Systems, Minneapolis, MN). Confluent proximal tubular cells were stimulated with 5 mg/ml delipidated bovine serum albumin (dBSA; Sigma) or 1 μM AngII in the presence or absence of PTX.

**Rat Interstitial Fibroblast Culture**

Normal rat kidney fibroblast cells (NRK-49F) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were cultured in DMEM supplemented with 10% FCS at 37°C, 5% CO₂ in a humidified incubator. Confluent cells were made quiescent by placing them in DMEM supplemented with 0.5% FCS for 24 h. They were then stimulated with 1 μM AngII or 5 ng/ml TGF-β1 in the presence or absence of PTX.

**Assessment of Cell Proliferation**

A modified MTT assay was used to measure cell proliferation as described previously (18). Briefly, NRK-49F fibroblasts were grown in 96-well microplates in density of 4000 cells/well in DMEM with 10% FCS. After 24-h incubation, cells were added PTX or vehicle. Final 10% MTT was added into every well in the last 4-h incubation. Finally, the medium was aspirated, and 100% ethanol was added to release the chromogen. Absorbance at the reference wavelength of 630 nm and test wavelength of 570 nm was measured with an ELISA.

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**Figure 1.** Pentoxifylline (PTX) attenuated the renal disease progression in rats with remnant kidney independent of changes in BP. Rats received either vehicle or pentoxifylline from day 7 to week 8 after surgery for renal mass reduction (RMR/VEH and RMR/PTX, n = 16, respectively). A parallel study was conducted in rats after sham operation (SHM/VEH and SHM/PTX, n = 16, respectively). Eight rats from each group were sacrificed on weeks 2 and 8, respectively. (A) Body weight increased in all groups throughout the study but was lower in RMR rats. (B and C) PTX reduced the progressively elevated levels of urinary protein excretion and plasma creatinine in RMR rats. (D) PTX did not change the elevation of mean aortic BP. Data are expressed as mean ± SD; n = 16 on week 1, and n = 8 for each group at later time points. *P < 0.05 versus SHM rats; #P < 0.05 versus RMR/VEH at the corresponding time points.
reader. The optic density obtained from modified MTT assay had good correlation with the cell number.

**Total RNA Extraction and Northern Blot Analysis**

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (18). The concentration of each sample was determined using spectrophotometry with the absorbance at 260 nm ($A_{260}$). The purity of each sample was determined on the basis of the ratio of $A_{260}$ to $A_{280}$. Total RNA was electrophoresed on formaldehyde-denatured 1% agarose gels in MOPS buffer (0.2 M morpholinepropanesulfonic acid, 0.05 M sodium acetate, 0.01 M EDTA), and subsequently transferred to nylon membranes. Hybridization was performed with digoxigenin-labeled RNA probes. The blots were developed using CSPD (Roche, Germany) as the substrate for alkaline phosphatase and normalized against the signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages.

Human type I (α1) collagen, type III (α1) collagen, rat TGF-β1, monocyte chemoattractant protein-1 (MCP-1), and GAPDH RNA probes were synthesized as previously stated in Materials and Methods (22). For synthesis of rat platelet-derived growth factor (PDGF) B chain, fibroblast growth factor-2 (FGF-2), connective tissue growth factor (CTGF) RNA probes, cDNA fragment was first generated by reverse transcription-PCR (RT-PCR) from the total RNA of rat mesangial cells or NRK-49F fibroblasts, using the following specific primer pairs: PDGF B chain, upstream 5'-TTGTGAGAAAGAAGCCAGTC-3' (corresponding to bases 449 to 468) and downstream 5'-TGTGCTTAAACTTTCGGTG-3' (corresponding to bases 642 to 661); FGF-2, upstream 5'-TCGGTCTCTCGGCTTCAGGC-3' (corresponding to bases 984 to 1003); CTGF, upstream 5'-AGAGAGTGCTAGGTCTTGGG-3' (corresponding to bases 361 to 380) and downstream 5'-CTAATATCTCAGTTCCAGGC-3' (corresponding to bases 559 to 578) and downstream 5'-GGCTTAAAATGCGCCAAGCCTGTG-3' (corresponding to bases 995 to 1014) (25–27). The products were subsequently subcloned into the pGEM-dT vectors (Promega, Madison, WI). The cloned cDNA was then linearized and used as the templates for in vitro transcription of antisense digoxigenin-conjugated riboprobes, following the supplier’s instruction.

**Miscellaneous Measurements**

Blood samples were collected using EDTA-rinsed syringes. Plasma was obtained after centrifugation and kept frozen in aliquots at −70°C until assayed. Twenty-four–hour urine samples were filtered and kept frozen in aliquots at −70°C until assayed. Creatinine of plasma and urine was measured using the alkaline picrate method in the Department of Laboratory Medicine in our institute. Protein concentration of urine was determined by BioRad protein assay (BioRad, Hercules, CA).

**Statistical Analyses**

Statistical analyses were carried out using SPSS/Windows (SPSS Inc.) software on a personal computer. The statistical significance was evaluated by one-way ANOVA using the Bonferroni correction application.

**Results**

**Pentoxifylline Attenuated the Renal Disease Progression in Rats with Remnant Kidney Independent of Changes in BP**

In the first set of animal experiments studying the effects of PTX on the renal disease progression in rats with remnant kidney, the body weight increased in all groups throughout the study, but was lower in RMR rats (Figure 1A). RMR rats developed progressively higher levels of urinary protein excretion and plasma creatinine, in comparison with SHM rats from week 1 after surgery. The levels of urinary protein excretion and plasma creatinine were comparable in RMR rats before they started to receive either vehicle or PTX (Figure 1, B and C). These levels were also comparable in SHM rats before the drug treatment. PTX did not affect the levels of urinary protein excretion and plasma creatinine in SHM rats. Intriguingly, PTX consistently reduced 60% of the elevated urinary protein excretion by RMR rats (Figure 1B) and 40% of the elevated plasma creatinine (Figure 1C). However, the hypertension that developed in RMR rats was not affected by PTX (Figure 1D).

The assessment of renal cortex of vehicle-treated RMR rats developed in RMR rats was not affected by PTX (Figure 1D). The combined therapy of PTX+CLZ alone could attenuate the glomerulosclerosis and tubulointerstitial damage in RMR rats. The combination of PTX+CLZ further attenuated the renal pathology to the levels similar to those found in SHM rats. The results of assessment are shown in Figures 3 and 9. Magnification: ×400. Calibration bars: 25 μm in length.

Figure 2. PTX and cilazapril (CLZ) ameliorated the renal pathology in rats with remnant kidney. Shown are the light microscopy of periodic acid–Schiff–stained sections of the renal cortex on week 8 after surgery, for SHM rats given vehicle or PTX (SHM/VEH, SHM/PTX) and RMR rats given PTX (RMR/PTX), cilazapril (RMR/CLZ), or PTX+CLZ (RMR/PTX+CLZ) from day 7. Either PTX or CLZ alone could attenuate the glomerulosclerosis and tubulointerstitial damage in RMR rats. The combined therapy of PTX+CLZ further attenuated the renal pathology to the levels similar to those found in SHM rats. The results of assessment are shown in Figures 3 and 9. Magnification: ×400. Calibration bars: 25 μm in length.
function, PTX also reduced the increased glomerular cellularity and volume by 30 to 40% as well as attenuated glomerulosclerosis and tubulointerstitial damage by 40 to 50% in RMR rats (Figures 2 and 3).

These data demonstrated that PTX could attenuate the renal disease progression in rats with remnant kidney. We further investigated the possible mechanisms for the renoprotective effect of PTX.

Pentoxifylline Attenuated the Interstitial Inflammation in Remnant Kidney

Attenuation of tubulointerstitial damage by PTX led us to study its effects on the interstitial inflammation in remnant kidney. Increasingly high numbers of cells expressing MHC class II antigen, macrophages expressing ED-1 antigen, and lymphocytes expressing CD4 or CD8 glycoprotein accumulated into the cortical interstitium in RMR rats from week 2 after surgery while being reduced by 40 to 60% by PTX (Figures 4 and 5).

These data suggest that PTX may reduce the interstitial infiltration of inflammatory mononuclear cells and cells expressing MHC class II antigen in remnant kidney, which should be responsible for the attenuation of tubulointerstitial damage.

Pentoxifylline Downregulated MCP-1 Gene Expression

Expression of MCP-1 is increased in the renal cortex of RMR rats, and the pathologic significance of MCP-1–recruited inflammatory mononuclear cells is demonstrated in experimental tubulointerstitial nephritis (5,28,29); we therefore examined the effect of PTX on the expression of MCP-1 gene. We found threefold increases in MCP-1 mRNA levels in the cortex of remnant kidney on weeks 2 and 8 after surgery, and the increases were reduced by 60% by PTX (Figure 6A).

Proximal tubular cells, when stimulated by albumin, can produce MCP-1 (30). Furthermore, the expression of MCP-1 in proximal tubular cells remnant kidneys is reduced after ACEI or ARB treatment, suggesting the role of AngII in triggering MCP-1 (5). We therefore examined the effect of PTX on the MCP-1 gene expression in proximal tubular cells stimulated with albumin or ANG II. Confluent proximal tubular cells were stimulated with 5 mg/ml dBSA or 1 μM AngII for 8 h in the presence or absence of PTX. The levels of MCP-1 mRNA were increased 3.6-fold by dBSA and 2.2-fold by AngII. PTX was found to reduce the upregulation of MCP-1 gene in a dose-dependent manner (Figures 6B and C).

Altogether, PTX was found to reduce the upregulation of MCP-1 gene in the cortex of remnant kidney and in cultured proximal tubular cells stimulated with dBSA or AngII, suggesting that PTX can suppress inflammatory cells recruitment induced by either proteinuria or AngII after renal ablation.

Pentoxifylline Reduced the Numbers of Interstitial Myofibroblasts and the Expression of Mitogenic and Profibrogenic Genes in Remnant Kidney

Having demonstrated that PTX attenuated the tubulointerstitial damage, we next examined the effect of PTX on interstitial myofibroblasts characterized by the expression of
Figure 4. PTX and CLZ ameliorated the interstitial inflammation and accumulation of myofibroblasts in rats with remnant kidney. Shown are the light microscopy of immunopathology of the renal cortex on week 8 after surgery for SHM rats given vehicle (SHM) and RMR rats given PTX (RMR/PTX), CLZ (RMR/CLZ), or PTX+CLZ (RMR/PTX+CLZ) from day 7. Immunostaining was performed for the detection of ED1+ macrophages, CD4+ lymphocytes, CD8+ lymphocytes, MHC II+ cells, and α-SMA+ myofibroblasts. Cells positive for specific antigen were brown-stained. In comparison with RMR/VEH, PTX or CLZ reduced the numbers of interstitial cells expressing ED1, CD4, CD8, MHC II, or α-SMA. The combined therapy of PTX+CLZ further reduced the numbers of cells expressing specific antigen. The results of assessment are shown in Figures 5 and 9. Magnification: ×400. Calibration bars: 25 μm in length.
**Figure 4.** (continued)
α-SMA. α-SMA was essentially expressed in vascular smooth muscle cells in SHM rats. In contrast, a high number of cells with α-SMA expression surrounding the peritubular and periglomerular spaces developed with time in RMR rats after surgery (Figure 4). PTX was found to reduce the numbers of α-SMA–positive cells in cortical interstitium of RMR rats (RMR/VEH versus RMR/PTX on week 8, 13.7 ± 4.2 versus 5.2 ± 2.9 cells/0.0625 mm²; *P < 0.05).

PTX was found to reduce the numbers of interstitial myofibroblasts and attenuate the interstitial fibrosis; therefore, we further examined the expression of mitogenic genes for the proliferation of fibroblasts and profibrogenic genes implicated in the renal fibrosis in the cortex of RMR rats, as well as the effect of PTX on these genes. Through the Northern blot analyses of cortical RNA, we found twofold to fourfold increases in the mRNA levels of PDGF, FGF-2, TGF-β1, CTGF, and types I and III collagen (α1) in RMR rats on week 8. However, PTX reduced the upregulation of these genes by 50 to 60% (Figure 7).

These data indicate that PTX can downregulate the gene expression for fibroblast proliferation and renal fibrosis, as well as reduce the numbers of interstitial myofibroblasts in the renal cortex of RMR rats, which may lead to the amelioration of interstitial fibrosis.

**Pentoxifylline Suppressed Proliferation and Profibrogenic Gene Expression of Interstitial Fibroblasts and Glomerular Mesangial Cells**

Several lines of evidence suggest that PTX can suppress the cell proliferation and collagen secretion in interstitial fibroblasts and glomerular mesangial cells (18–20). We therefore examined the effect of PTX on the gene expression of TGF-β1 and the downstream mediator, CTGF, induced by AngII, as well as the effect of PTX on the gene expression of CTGF induced by TGF-β1 in either NRK-49F or mesangial cells. At concentrations from 0.1 to 1 mM, the addition of PTX to cell cultures induced a dose-dependent inhibition on the cell proliferation of serum-stimulated NRK-49F assayed using the modified MTT method (Figure 8A). Northern blot analyses revealed a 2.5-fold increase for TGF-β1 mRNA and a fivefold increase for CTGF mRNA in quiescent NRK-49F incubated with 1 μM AngII for 4 h. The increase sustained to 24 h after stimulation, when the experiment was finished. PTX treatment...

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**Figure 5.** PTX attenuated the interstitial inflammation in remnant kidney. Eight rats from each group (SHM/VEH, SHM/PTX, RMR/VEH, and RMR/PTX) were sacrificed for immunostaining of ED1+ macrophages, CD4+ lymphocytes, CD8+ lymphocytes, and MHC II+ cells on weeks 2 and 8 after surgery, respectively. Cells positive for specific antigen in each group of rats are expressed as mean ± SD per 0.0625 mm² ocular grid in high-power microscopic fields (×400) of the cortical interstitium. *P < 0.05 versus SHM rats; †P < 0.05 versus RMR/VEH at the corresponding time points.

**Figure 6.** PTX downregulated the expression of monocyte chemoattractant protein-1 gene. (A) PTX downregulated the expression of MCP-1 gene in RMR rats. Eight rats from each group (SHM/VEH, SHM/PTX, RMR/VEH, and RMR/PTX) were sacrificed to analyze the expression of MCP-1 gene on week 2. The signals of MCP-1 are normalized against those of 18S rRNA. Representative of Northern blot analysis is one of eight results in the parallel groups. The data in bar charts are expressed as mean ± SD from the eight rats in each group. *P < 0.05 versus SHM rats; †P < 0.05 versus RMR/VEH; (B and C) PTX downregulated MCP-1 gene expression in proximal tubular cells stimulated with delipidated BSA (dBSA) or angiotensin II (AngII). Confluent proximal tubular cells treated by PTX of varying concentrations were stimulated with 5 mg/ml dBSA or 1 μM AngII for 8 h. Representative of Northern blot analysis is one of three independent experiments with similar results. The signals of MCP-1 are normalized against those of 18S rRNA.
did not reduce the upregulation of TGF-β₁ induced by AngII, but it remarkably attenuated the expression of CTGF (Figure 8B). CTGF is a downstream mediator for the fibrogenic effect of TGF-β₁ in mesangial cells and fibroblasts (31–33); therefore, we further elucidated the effect of PTX on the gene expression of CTGF induced by TGF-β₁. Increased mRNA levels of CTGF were observed in NRK-49F after stimulation with TGF-β₁ for 4 to 24 h. PTX treatment was found to

Figure 7. PTX downregulated the expression of mitogenic and profibrogenic genes in remnant kidney. Eight rats from each group (SHM/VEH, SHM/PTX, RMR/VEH, and RMR/PTX) were sacrificed to analyze the gene expression. Northern blot analysis was performed for platelet-derived growth factor B chain (PDGF-B), fibroblast growth factor-2 (FGF-2), transforming growth factor-β₁ (TGF-β₁), connective tissue growth factor (CTGF), type I collagen [col I (α1)], and type III collagen [col III (α1)]. The signals of these growth factor and collagen genes were normalized against those of GAPDH, respectively. Representative of Northern blot analysis for each gene is one of eight results in the parallel groups (A). The data in bar charts are expressed as mean ± SD from the eight rats in each group (B). *P < 0.05 versus SHM rats; #P < 0.05 versus RMR/VEH.
CTGF gene expression in interstitial fibroblasts stimulated by TGF-
the presence or absence of PTX for 4 h. (C) PTX downregulated
/H9262
 Quiescent NRK-49F fibroblasts were stimulated with 1
CTGF but not TGF-
/H9252
 vehicle-treated. (B) PTX downregulated the gene expression of
versus
/P
in independent experiments performed in quadruplicate. *
/H9252
 proliferation, but it also reduces AngII-induced and TGF-
was not affected by PTX (data not shown).

decrease the expression of CTGF stimulated by TGF-\(\beta_1\) in a
dose-dependent manner (Figure 8C). Similar to the results
observed in NRK-49F, PTX also downregulated the mRNA
levels of CTGF in rat mesangial cells induced by either AngII
or TGF-\(\beta_1\), however, AngII-induced expression of TGF-\(\beta_1\)
was not affected by PTX (data not shown).

These data indicate that PTX not only suppresses the cell
proliferation, but it also reduces AngII-induced and TGF-\(\beta_1\)-
induced CTGF gene expression in cultured NRK-49F and
glomerular mesangial cells, which will result in the ameliora-
tion of glomerulosclerosis and interstitial fibrosis.

Discussion

This study demonstrated that PTX, a clinically available
phosphodiesterase inhibitor, attenuated the renal disease pro-
gression in rats with remnant kidney through its effects against
cell proliferation, inflammation, and fibrosis. In particular, the
therapy combining PTX with CLZ dramatically attenuated proteinuria and renal pathology to the levels similar to those found in SHM rats and lowered plasma creatinine to the level comparable to that of RMR rats before any treatment on day 7 (Figure 9, A, B, D, and E).

In comparison with RMR rats given only vehicle, CLZ or
PTX alone comparably reduced the numbers of interstitial cells expressing ED1, CD4, CD8, MHC class II antigen, or \(\alpha\)-SMA
(Figures 4 and 9, F through J). Interestingly, PTX reduced the
numbers of cells expressing MHC class II antigen more mark-
dedly than CLZ did (Figure 9I). The therapy combining PTX with CLZ almost completely abolished the interstitial infiltra-
tion of macrophages/lymphocytes as well as the overexpression of MHC class II antigen in RMR rats (Figure 9, F through
I).

These data suggest that the therapy combining PTX with
CLZ has the potential to prevent the renal disease progression almost completely in rats with remnant kidney.

Because of uncontrolled hypertension and residual protein-
uria seen in RMR rats receiving PTX, the combination of PTX
with an agent that could attenuate hypertension and protein-
uria, such as an ACEI or ARB, may have the potential to
further prevent renal disease progression. We therefore com-
pared the effects of combining PTX with CLZ versus either
drug alone on the renal disease progression in RMR rats.
Elevated levels of proteinuria, plasma creatinine, and increased
glomerulosclerosis were lessened more in RMR rats given
CLZ than PTX alone (Figure 9, A, B, and D). Furthermore,
CLZ had a remarkable antihypertensive effect maintaining the
BP similar to that in SHM rats (Figure 9C). There was, how-
ever, no difference in tubulointerstitial damage score in RMR
rats given either CLZ or PTX (Figure 9E). The therapy com-
bining PTX with CLZ dramatically attenuated proteinuria and
renal pathology to the levels similar to those found in SHM rats
and lowered plasma creatinine to the level comparable to that
of RMR rats before any treatment on day 7 (Figure 9, A, B, D,
and E).

In the first set of experiments, we demonstrated that PTX
could attenuate the chronic renal disease progression, particu-
larly through a renoprotective effect that was largely indepen-
dent of changes in BP. The renal pathology of remnant kidney
is characterized with the progressive glomerulosclerosis and
interstitial fibrosis as well as the preceding hyperplasia of
glomerular mesangial cells and interstitial fibroblasts
(3,5,8,10,11,34–36). These pathologic changes were amelio-
rated by PTX treatment in this study. Furthermore, we also
demonstrated that PTX could reduce the upregulation of PDGF
and FGF-2 genes in remnant kidneys, two important mitogens
for mesangial cells and fibroblasts (3,18,37). Several lines of
evidence indicate that PTX can suppress the proliferation of
glomerular mesangial cells and interstitial fibroblasts in vitro

Figure 8. PTX suppressed proliferation and profibrogenic gene ex-
pression of interstitial fibroblasts. (A) PTX suppressed the prolifera-
tion of interstitial fibroblasts. Varying concentrations of PTX were
added after overnight plating of NRK-49F fibroblasts and further
incubated for 48 h. Data are expressed as mean ± SD for three
independent experiments performed in quadruplicate. *\(P < 0.05\)
versus vehicle-treated. (B) PTX downregulated the gene expression of
CTGF but not TGF-\(\beta_1\) in interstitial fibroblasts stimulated by AngII.
Quiescent NRK-49F fibroblasts were stimulated with 1 \(\mu M\) AngII in
the presence or absence of PTX for 4 h. (C) PTX downregulated
CTGF gene expression in interstitial fibroblasts stimulated by TGF-
\(\beta_1\). Quiescent NRK-49F fibroblasts stimulated with 5 ng/ml TGF-\(\beta_1\)
were treated with PTX of varying concentrations for 4 h. The signals
of CTGF were normalized against those of GAPDH. Representatives
of Northern blot analysis in panels B and C are one of three indepen-
dent experiments with similar results, respectively.
Figure 9. Therapy combining PTX with CLZ prevented renal disease progression almost completely in rats with remnant kidney. RMR rats received VEH, PTX, CLZ, or PTX+CLZ from day 7 to week 8 after surgery (n = 12 for each group). SHM rats given VEH were used as normal controls (n = 8). All rats were sacrificed to examine (A) urinary protein excretion, (B) plasma creatinine, (C) mean aortic BP, (D and E) renal pathology, and (F through J) renal immunopathology. The combined therapy PTX+CLZ attenuated proteinuria, renal pathology, and immunopathology to levels similar to those found in SHM rats. The combined therapy also lowered the plasma creatinine to the level comparable to that of RMR rats before any treatment on day 7 (Figure 1C). Data in bar charts are expressed as mean ± SD from the rats in each group. Cells positive for specific antigen in renal immunopathology are expressed as cells per 0.0625 mm² ocular grid in high-power microscopic fields (×400) of the cortical interstitium in each group. α, P < 0.05 versus SHM; β, P < 0.05 versus RMR/VEH; γ, P < 0.05 versus RMR/CLZ; δ, P < 0.05 versus RMR/PTX.
or in vivo (18–20,22). Therefore, one of the possible mechanisms for the renoprotection may be linked to the effect of PTX against the proliferation of glomerular mesangial cells and interstitial fibroblasts.

High numbers of inflammatory mononuclear cells and cells expressing MHC class II antigen in the cortical interstitium of remnant kidney in this study were in accordance with previous studies (5,8,10,34–36). The pathologic significance of interstitial inflammation has been well recognized and further strengthened by the evidence that mycophenolate mofetil can attenuate the renal injury in rats with remnant kidney (8,10,11). In this study, we found that PTX could attenuate the infiltration of macrophages and lymphocytes in remnant kidney. Such attenuation could be in part due to the effect of PTX against the proliferation of inflammatory mononuclear cells (16). However, we also found that PTX could reduce the upregulation of MCP-1 gene in the remnant kidney and in the albumin-stimulated or AngII-stimulated proximal tubular cells. Recent studies have shown that the increased expression of MCP-1 in proximal tubular cells and interstitial mononuclear cells of the remnant kidney is downregulated after ACEI or ARB treatment (5). Albumin can stimulate proximal tubular cells to produce MCP-1 through nuclear factor-κB (NF-κB) activation (30).

Furthermore, there are studies demonstrating that AngII may stimulate MCP-1 to recruit inflammatory mononuclear cells through NF-κB–dependent pathway (38,39). PTX is reported to be an inhibitor of NF-κB and inhibits MCP-1 production in macrophages/monocytes (40,41). Therefore, the attenuation of interstitial inflammatory cell infiltration by PTX may be also due to the downregulation of MCP-1 in remnant kidney. In addition, we also found that PTX could reduce the overexpression of MHC class II antigen in the remnant kidney. The overexpression of MHC class II antigen has been known to increase the activation of T lymphocytes by macrophages and lead to tubulointerstitial damage (8,36). The inhibitory effect of PTX on MHC class II antigen expression may be partially due to the decreased infiltration of macrophages/monocytes in the remnant kidney. However, it is also possibly due to the inhibition of MHC class II antigen expression in macrophages by PTX (42,43). Therefore, the second possible mechanism to prevent the renal disease progression may be due to the effect of PTX on attenuating inflammation through reducing the overexpression of MHC class II antigen and MCP-1 in the remnant kidney.

A variety of cytokines, including PDGF, FGF-2, TGF-β1, and CTGF, are important growth factors for cell proliferation and extracellular matrix production in glomerular mesangial cells and interstitial fibroblasts through paracrine or autocrine stimulation (3,31–33,37). In this study, we found that PTX could downregulate the gene expression of these growth factors by 50 to 60% in the remnant kidney, though it could not affect the expression of TGF-β1 gene in either glomerular mesangial cells or interstitial fibroblasts stimulated by AngII. PTX has been reported to reduce FGF-2 gene expression in serum-stimulated renal fibroblasts, but there seems to be no evidence that PTX can inhibit PDGF and TGF-β1 gene expression in cultured cells (19). We therefore suggest that PTX downregulated the gene expression of PDGF, FGF-2, and TGF-β1 in the remnant kidney in part due to the decreased numbers of cells secreting these cytokines, including infiltrating inflammatory cells, glomerular mesangial cells, and interstitial myofibroblasts. PTX may prevent the progressive renal fibrosis by reducing the upregulation of these growth factor and collagen genes in the remnant kidney, which is the third possible mechanism for its renoprotective effect. However, the inhibition of TGF-β1 signaling and suppression of cellular immunity by PTX may impair the mechanisms for defense and healing in surgical wound (44). This may probably explain why we observed poor healing of surgical wound and higher mortality in rats receiving PTX from the day of surgery in the pilot study.

CLZ alone prevented renal disease progression remarkably, but the therapy combining CLZ with PTX provided more pronounced protection that halted the renal disease progression almost completely. The result suggests that such a combined therapy may have additional effects. Our observation was similar to the outcome of rats with remnant kidney receiving the therapy combining ACEI with mycophenolate mofetil (8). However, PTX is a drug with few clinical side effects, especially sparing bone marrow suppression.

In conclusion, PTX alone can attenuate the progression of chronic renal disease through its effects against cell proliferation, inflammation, and fibrosis. Furthermore, its combination with CLZ may have the potential to halt renal disease progression.

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