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Pirfenidone improves renal function and fibrosis in the post-obstructed kidney

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Pirfenidone improves renal function and fibrosis in the post-obstructed kidney.

Background. Pirfenidone™ (PFD) is a novel anti-fibrotic agent that can prevent and even reverse extracellular matrix accumulation in several organs, as shown by experimental and clinical studies. Unilateral ureteral obstruction (UUO) is a well-characterized model of experimental renal disease culminating in tubulointerstitial fibrosis.

Methods. UUO or sham-operated rats were administered PFD (500 mg/kg/day) in their food for 21 days to examine the effect on collagen production. The renal function was measured in the kidney after release of obstruction which had been maintained for one week to examine the effects of PFD on restoration after renal dysfunction.

Results. The collagen content detected by hydroxyproline progressively increased in kidney with UUO for 21 days. These increases were significantly suppressed by administration of PFD. PFD had no effect on collagen production in sham-operated rats. Expression of mRNA for type IV and I collagen and matrix metalloproteinase-2 in the cortex increased with UUO, but was inhibited by PFD treatment. The levels of cortical transforming growth factor-β (TGF-β) mRNA progressively rose with UUO for 21 days, but this increase also could be suppressed by PFD. Inulin clearance of the obstructed kidney was markedly depressed and remained low at five weeks after release. A progressive increase in hydroxyproline content was also observed in the post-obstructed kidney despite the release of obstruction. Administration of PFD following the release not only attenuated collagen accumulation, but also induced recovery of the impaired renal function.

Conclusions. These results demonstrate that PFD can attenuate both renal fibrosis and renal damage in this model, and suggest that PFD can be clinically useful for preventing progressive, irreversible renal failure.

In recent years, decreases in the glomerular filtration rate (GFR) due to different primary renal diseases have been shown to be closely correlated to the degree of changes in the tubulointerstitial area. Irrespective of the underlying cause, many kidney diseases lead to tubulointerstitial inflammation and eventual interstitial fibrosis with permanent loss of renal function [1–5]. Most medical investigators agree that new therapeutic strategies should be targeted at developing effective methods for inhibiting renal fibrogenesis. Although their therapeutic potential is still under investigation, anti-fibrotic therapy holds great promise for the prevention of renal disease that can progress to end-stage renal failure.

Pirfenidone® (PFD) is a newly developed compound that can prevent and even reverse extracellular matrix (ECM) accumulation in several organs, as shown by experimental studies of pulmonary fibrosis [6] and peritoneal sclerosis [7]. In patients with end-stage pulmonary fibrosis, PFD not only restored significant pulmonary function, but also improved the survival rate. We have recently shown that PFD can effectively prevent the development of progressive renal disease in the monoclonal anti-Thy-1 antibody [8] and the remnant kidney [9] rat model.

One well-characterized model of experimental renal disease culminating in tubulointerstitial fibrosis uses chronic unilateral ureteral obstruction (UUO) [10–15]. The impairment has been described as reversible at the early stage [16]. However, prolonged obstruction frequently leads to renal interstitial fibrosis and progressive destruction of the kidney [17]. Although the renal dysfunction during or following the release of obstruction has been linked to deterioration of the renal vasoacting system [18, 19], the mechanisms responsible for UUO-induced kidney fibrosis are not well understood.

In the present study, to find further evidence of the efficacy of PFD in chronic renal failure (CRF), we investigated the effect of PFD on the development of tubulointerstitial fibrosis induced by UUO. Furthermore, we endeavored to determine whether inhibition of interstitial fibrosis by an anti-fibrotic agent could significantly contribute to an effective restoration of impaired renal function resulting from fibrosis. We therefore tried to find whether renal function could be improved by PFD treatment with of rats with post-obstructed kidneys.

Key words: obstructive nephropathy, anti-fibrotic therapy, TGF-β, extracellular matrix accumulation, tubulointerstitial disease, unilateral ureteral obstruction.

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METHODS

Animal study

Male Sprague-Dawley rats (8 weeks old at the start of the experiment) were used in the study. Under light anesthesia with pentobarbital, complete ureteral obstruction of the left kidney was produced by ligating the ureter near the bladder with a 4-0 silk suture through a small abdominal incision. Sham-operated rats had their ureters manipulated but not ligated. The incision was closed, and the animals were allowed free access to tap water and standard chow with or without PFD. In some experiments, administration of PFD was started seven days subsequent to the obstruction. PFD was administered by an admixture in the food at a concentration (0.6 to 0.9%) estimated to deliver approximately 500 mg/kg/day of PFD. Rats with UUO were sacrificed under pentobarbital anesthesia after 3, 7, 14, and 21 days from the start of the study.

After one week following UUO, the obstruction was released in another series of rats. The suprapubic suture was removed under light anesthesia. The ureteral obstruction was released by inserting a PE-50 catheter above the ligature of the left ureter made one week earlier, and the other end of the catheter was placed into the bladder to drain urine. The incision was again closed, and the animals were allowed free access to water and food with or without PFD until the clearance studies were performed.

Clearance study

Clearance studies were performed at one, two and five weeks after the release of the occlusion as previously described [20, 21]. On the day of the clearance study, each rat was anesthetized with pentobarbital (40 mg/kg body wt, i.p.) and kept on a heated table. A femoral artery and vein were cannulated with PE-50 tubing for blood sampling and measurement of arterial pressure and for supplemental infusion of solution, respectively. Urine samples from the obstructed and/or contralateral kidney were obtained respectively from each side, which cut the transplanted ureteral catheter into two pieces. Urine volume was determined by weighing the urine collected in pre-weighed tubes. A priming dose (20 mg/kg body wt) of inulin was given as a bolus into the femoral vein, followed by continuous infusion of 3% inulin at 30 μl/min. Samples from animals with mean arterial pressure of less than 85 mm Hg were excluded because they were considered to be aberrant. After an equilibration period, two consecutive 15-minute urine specimens were collected. The mean of these samples was used as a representative value for each animal. Arterial blood samples were obtained at the midpoint of each clearance period to determine the inulin concentrations. Inulin concentration in plasma and urine samples were measured by fluorometry using a previously described method [22]. At the end of the experiment, each rat was euthanized with an anesthetic overdose, and the kidney was rapidly removed and subjected to measurement of collagen content and morphometric examination.

Biochemical study

Collagen accumulation was evaluated for up to 21 days of UUO. On the day of examination, the rats were anesthetized with pentobarbital. The kidneys were removed and sectioned longitudinally. After the medulla was removed, the kidney was bisected with a sagittal cut. Half of the cortex was used for determination of hydroxyproline and protein, and the other half for extraction of total RNA.

Determination of hydroxyproline and protein

The collagen content in the renal cortex was assessed by determining total hydroxyproline according to the method of Wessner [23]. Briefly, the samples were suspended in 6 N HCl and hydrolyzed for three hours at 130°C in tightly capped tubes. The samples were then dried and resuspended in citrate-acetate buffer (pH 6.0). The samples were oxidized by chloramine T and mixed with *p*-dimethylamino-benzaldehyde, then the absorbance at 557 nm was measured. Hydroxyproline content was calculated from a standard curve constructed with hydroxy-L-proline. The results were expressed as hydroxyproline per mg protein.

Protein was determined according to Lowry et al [24] with bovine serum albumin as a standard.

Total RNA extraction and Northern blot analysis

Total RNA was extracted from the renal cortex by the acid-guanidinium thiocyanate-phenol-chloroform method [25]. Cortical tissue weighing 50 to 100 mg was dispersed at 4°C in 4 ml of 4 M guanidine thiocyanate, containing 0.5% sodium sarcosyl and 0.7% 2-mercaptoethanol, with a Polytron homogenizer. The homogenate was mixed with 0.4 ml of 2 M sodium acetate, pH 4.0, 4 ml of phenol, and 0.8 ml of chloroform-isoamylalcohol (49:1, vol/vol) and kept on ice for 20 minutes. The sample was centrifuged at 10,000 $\times$ g for 20 minutes, and the aqueous phase was subjected to phenol-chloroform extraction. RNA in the aqueous phase was precipitated with isopropanol, collected by centrifugation at 15,000 $\times$ g for 20 minutes, washed with 75% ethanol, solubilized in diethylpyrocarbonate-treated water, and stored at -70°C. The concentration of the RNA isolated was calculated on the basis of absorbance at 260 nm. Aliquots of total RNA, each consisting of 10 μg, were size-fractionated by electrophoresis in denaturing 1.0% agarose/2.2 M formaldehyde gels in 20 mM MOPS buffer, transferred overnight by capillary blotting in 10 $\times$ SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) to S&S Nytran nylon membrane (Schleicher & Schuell, Keene, NH, USA), and fixed to the membrane by exposure to UV.

Blots were prehybridized for four hours at 65°C in 5 $\times$ SSC, 5$\times$ Denhardt's solution (0.1% bovine serum albumin, Ficoll, and polyvinylpyrrolidine), 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 250 μg/ml sonicated salmon sperm DNA, and 50% formamide. Hybridization was performed

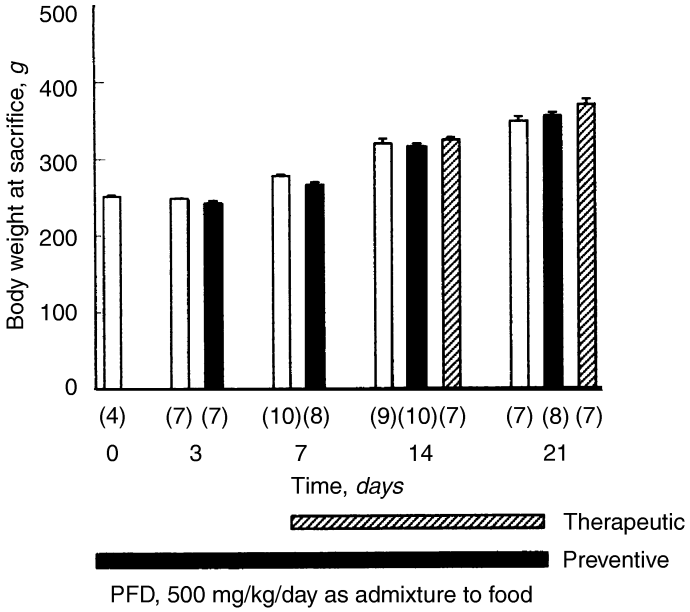
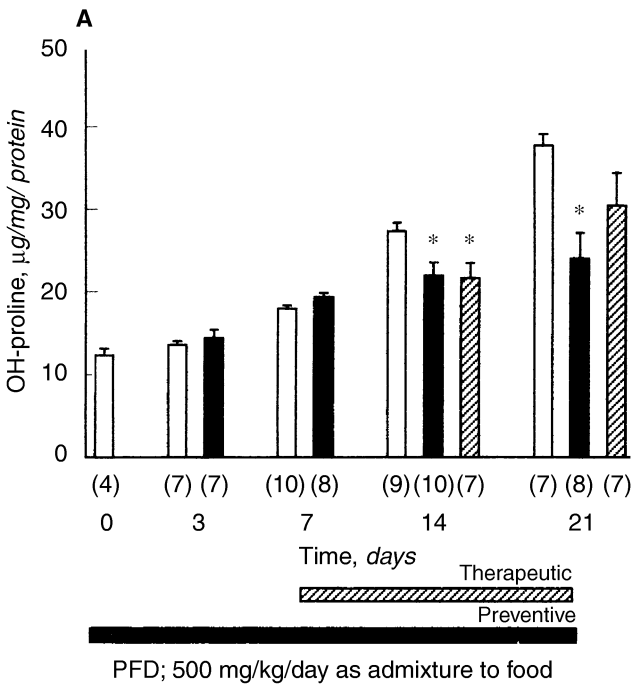


Fig. 1. Effect of Pirfenidone (PFD) on body weight in rats with ureteral obstruction. Data are represented as mean ± SE. The number of animals per group is shown in parenthesis below each bar. Symbols are: (□) unilateral ureteral obstruction (UUO) rats without PFD treatment; (■) UUO rats with PFD after the onset of obstruction; (▨) UUO rats with PFD from day 7 after obstruction.



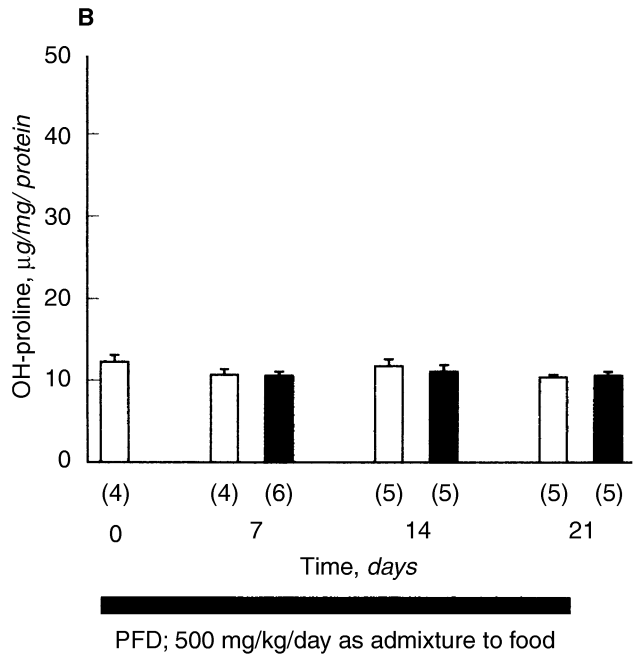


Fig. 2. Effect of Pirfenidone (PFD) on hydroxyproline content as the measurement of collagen in the kidney cortex of rats with ureteral obstruction (*A*) and sham-operated controls (*B*). Data are represented as mean ± SE. Symbols are: (□) unilateral ureteral obstruction (UUO) rats without PFD treatment; (■) UUO rats with PFD after the onset of obstruction; (▨) UUO rats with PFD from day 7 after obstruction. $^*P < 0.05$ versus untreated control group in each period. Numerals in parentheses show the number of animals.

for 24 hours at 65°C with a ^{32}P-labeled cDNA probe in a fresh prehybridization buffer supplemented with 10% dextran sulfate. The cDNA probes were labeled with [^{32}P]dCTP (Du Pont-New England Nuclear, Boston, MA, USA) to $>1 \times 10^8$ cpm/μg using a random primer labeling system (Gibco-BRL, Gaithersburg, MD, USA). Following hybridization, the membranes were washed once in 2 × SSC for 15 minutes at room temperature and twice in 0.2 × SSC with 0.1% SDS for 15 minutes at 65°C. The membranes were exposed to Kodak X-Omat X-ray films for one to two days at −70°C with an intensifying screen. The blots were stripped and reprobed with 28S or 18S ribosomal RNA using the conditions of labeling, hybridization, and washing as described above. The levels of mRNA were quantitated by densitometry using an optical scanner system (Fujix, BAS-2000II; Fuji Film Inc., Tokyo, Japan), normalized to that of 28S or 18S ribosomal RNA to correct for the difference in RNA loading and/or transfer.

The cDNAs used were a 1.3 kb *Pst*I/*Bam*HI fragment of Pα1R1 for rat α1 (I) collagen [26], a 2.1 kb *Eco*RI fragment of matrix metalloproteinase (MMP)-2 [27], and a 1.2 kb Bgl II fragment of TGF-β [28]. The portion of the major triple helical domain of the mouse α1(IV) chain encoding the 1.05 kb *Pst*I fragment [29] was subcloned and a 0.61 kb *Pst*I/*Puv*II fragment containing nucleotides 410-997 was used as the cDNA probe for type IV collagen (MUSOL4A1 from GenBank).

Histological study by light microscopy

Half of the kidney was fixed in phosphate-buffered formalin solution and processed in the usual manner. The sections up to 2 μm thick were embedded in paraffin wax and stained using routine techniques with PAM (periodic-acid-methenamine silver).

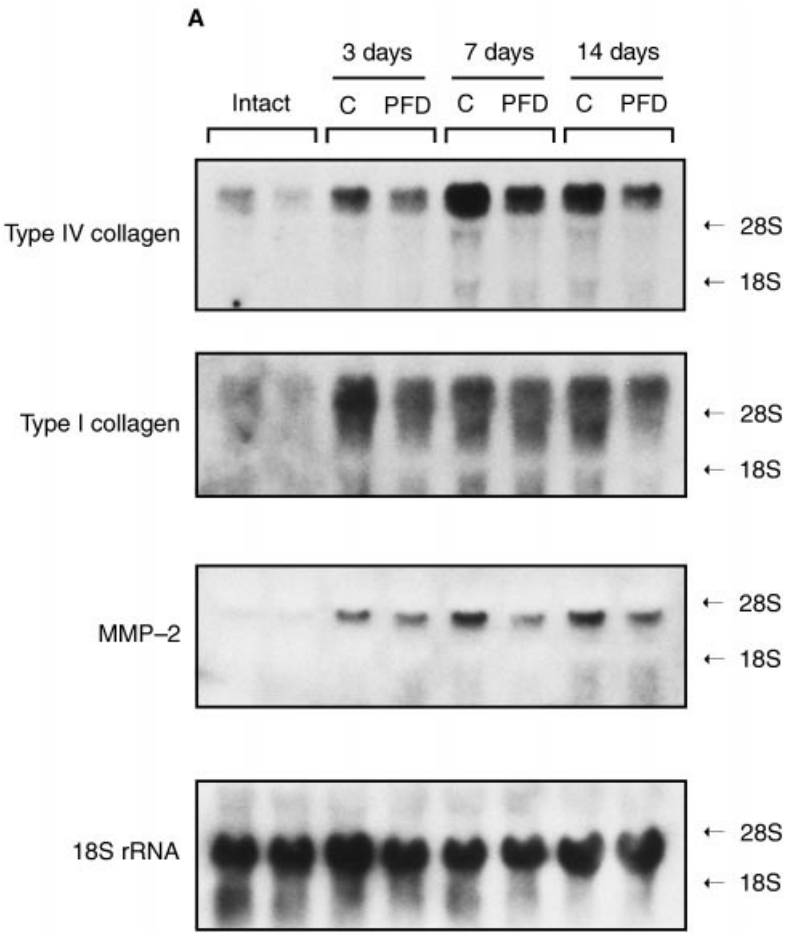


Fig. 3. Northern blot analysis of mRNA for type IV and I collagen, MMP-2 and 18S rRNA. (*A*) Typical autoradiograms for type IV (5.0 and 6.8 kb) and type I (4.7 and 5.7 kb) collagen and MMP-2 (3.1 kb) mRNA and 18S (1.9 kb) rRNA in the cortex of sham-operated rats (lanes 1 and 2), unilateral ureteral obstruction (UUO) for three days (lanes 3 and 4), UUO for seven days (lanes 5 and 6) and UUO for 14 days (lanes 7 and 8). UUO rats with PFD treatment (lanes 4, 6 and 8). (*B*) Quantitation of the mRNA. Data represent mean ± SE. Symbols are: (□) UUO rats without PFD treatment; (■) UUO rats with PFD after the onset of obstruction. $^*P < 0.05$ versus untreated control group in each period. Numerals in parentheses show the number of animals.

Statistical analysis

All results are expressed as mean ± SE. Statistical analysis was performed using Student's *t*-test for paired or unpaired samples as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Rates of weight gain were fairly comparable in all groups except for the first three days after operation when there was no weight gain (Fig. 1). There were no significant differences in the mean body weights for each time interval among the various groups, suggesting that total food intake was comparable in the groups for the entire study period.

Accumulation of collagen in renal cortex during unilateral ureteral obstruction

To determine the development of renal fibrosis, the amount of collagen in the renal cortex was quantitatively assessed by measuring the total content of hydroxyproline. Changes in this content after UUO are shown in Figure 2. In the obstructed kidney, the hydroxyproline content of the renal cortex increased progressively to 21 days (Day 0: baseline control, 12.3 ± 0.8 μg/mg protein). The increased collagen content was evident after only three days of obstruction. At 3, 7, 14 and 21 days after obstruction, the hydroxyproline content of the obstructed kidney increased to 110%, 146%, 222% and 306%, respectively. This increase could be significantly reduced by PFD at 14 and 21 days of UUO (178% and 183%, respectively). Even when treatment with PFD was delayed for seven days after obstruction, a significantly lower hydroxyproline content was observed at 14 days. Thus, progressive accumulation of collagen occurred in the renal cortex of the obstructed kidney and, in the late phase of UUO, this increase could be reduced by PFD. In contrast, PFD had no effect on the hydroxyproline content in the kidney of the sham-operated rats up to 21 days.

Expression of mRNA for type IV and I collagen, MMP-2 and TGF-β during UUO

To determine whether the increased collagen accumulation found in the obstructed kidney is associated with increases in mRNA levels for type IV and I collagen, Northern blot analysis was performed on total RNA prepared from the renal cortex. Results of a representative study are shown in Figure 3A. The mRNA levels for type IV collagen in the obstructed kidney were significantly higher than those at the level of Day 0 (baseline). The type

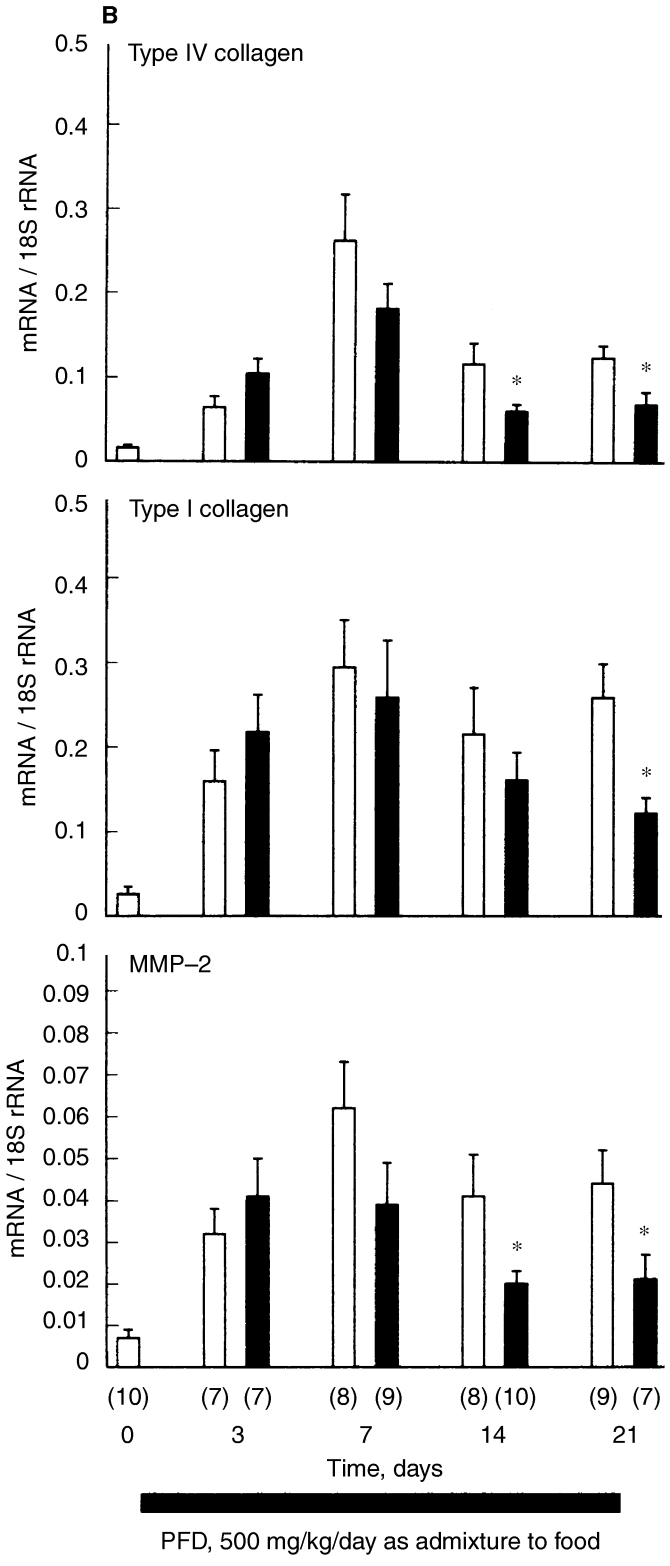


Fig. 3. *Continued.*

IV collagen mRNA expression reached maximum levels by seven days of UUO and then declined somewhat but remained higher than the baseline levels. In rats treated with PFD, increases in type IV collagen mRNA expression were significantly reduced at 14 and 21 days of UUO. Hybridization with the 18S rRNA control probe confirmed that equivalent amounts of RNA were loaded in each lane. The results of Northern analysis for mRNA were quantitated by densitometry, normalized to that of 18S rRNA (Fig. 3B). Likewise, expression of type I collagen mRNA was also increased in the obstructed kidney with a time course and effects of PFD comparable with that of type IV collagen mRNA. As shown by the representative example (Fig. 3A) and quantitalive analysis (Fig. 3B), type I collagen mRNA was increased by three days after initiation of UUO, peaking at seven days and remaining at a high level at 21 days after kidney obstruction. In PFD-treated animals, a significant reduction of increased type I collagen mRNA expression was observed at 21 days of UUO. These results demonstrate that in the obstructed kidney, mRNA levels for both type IV and I collagen increased to a maximum within three days of UUO and remained elevated until 21 days of UUO. Similar to the increases in hydroxyproline content, the increase in collagen mRNA expression was inhibited by PFD after 14 days, but not after seven days of UUO. Because evidence of a clearer response to PFD was observed in type IV collagen expression, our data suggest that the change in type IV collagen mRNA affords a greater contribution to the inhibition of collagen accumulation by PFD than that of type I collagen.

Since persistent accumulation of collagen occurring in the obstructed kidney might involve alterations in ECM-degrading pathways, we examined mRNA expression of matrix metalloproteinase (MMP)-2 (Fig. 3). The mRNA level of MMP-2 increased by UUO, which was in parallel with the data from collagen accumulation as well as the collagen mRNA data, and the expression reached its maximum by seven days after UUO. PFD showed effective inhibition at 14 and 21 days. These data suggested that collagen accumulation in UUO was independent of the inactivation of the collagen degrading system, and that PFD administration had no effect on this system in the present study.

To study the role of TGF-β in the regulatory process following UUO, we examined the expression of TGF-β mRNA. As shown in Figure 4, the mRNA levels for TGF-β in UUO rats increased remarkably in a manner similar to collagen accumulation. The levels in the obstructed kidney were significantly higher by three days and progressively increased at 21 days of UUO. Administration of PFD significantly inhibited increases in TGF-β mRNA after seven days of UUO. PFD was still effective even when treatment was delayed until seven days after initiation of UUO.

Collagen accumulation and functional recovery of the kidney following relief of UUO

Body and kidney weights in the clearance study are shown in Tables 1 and 2. No significant differences in body weight and contralateral kidney weight were found between

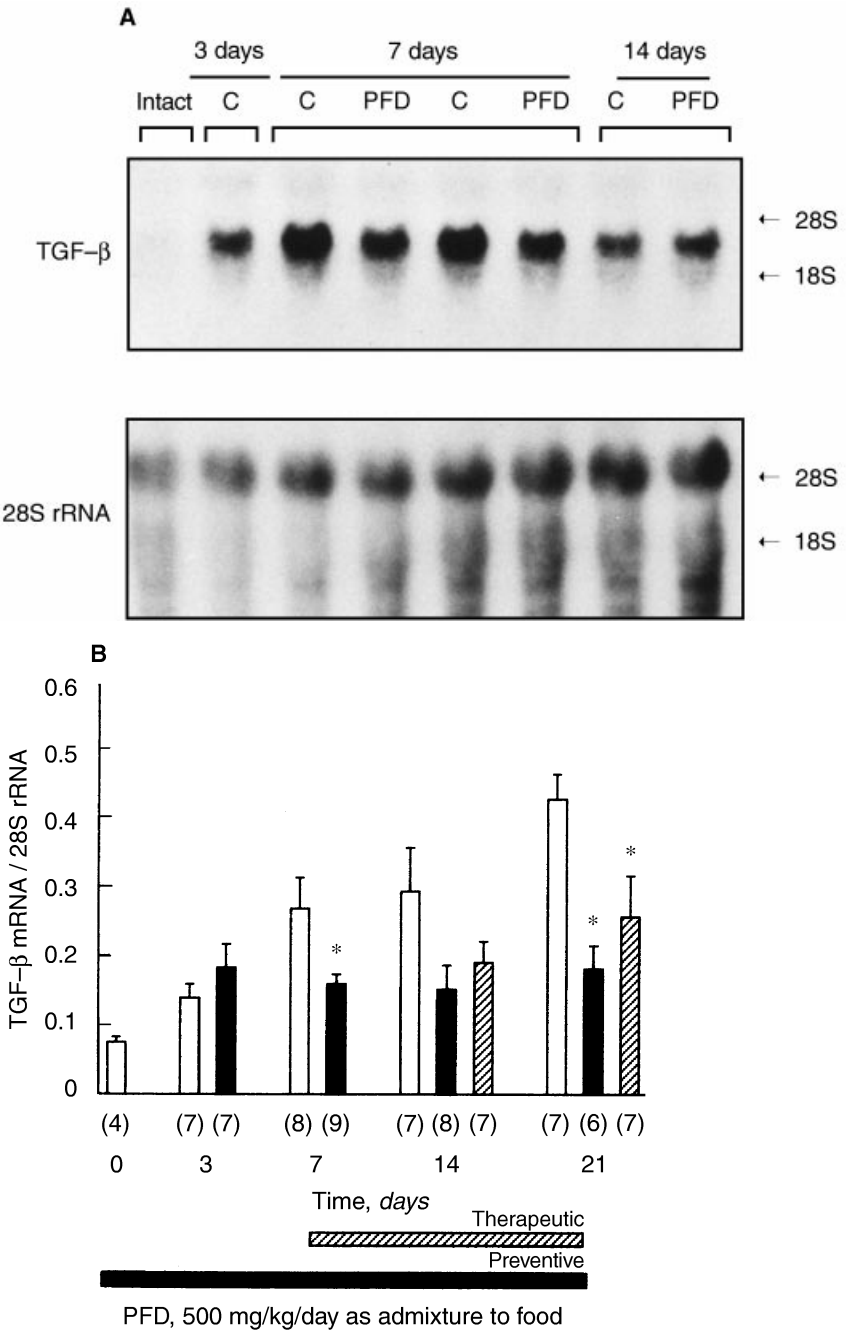


Fig. 4. Northern blot analysis of mRNA for TGF-β and 28S rRNA. (*A*) Typical autoradiograms for TGF-β (2.5 kb) mRNA and 28S (4.7 kb) rRNA in the cortex of sham-operated rat (lane 1), UUO for three days (lane 2), UUO for seven days (lanes 3, 4, 5 and 6) and UUO for 14 days (lanes 7 and 8). UUO rats with PFD treated (lanes 4, 6 and 8). (*B*) Quantitation of the mRNA. Data are represented as mean ± SE. Symbols are: (□) control; (■) preventive dose of PFD; (▨) therapeutic dose of PFD. $^*P < 0.05$ versus untreated control group in each period. Numbers in parentheses show the number of animals.

the UUO groups with or without PFD. However, the obstructed kidney in the UUO rats was significantly heavier than that of the contralateral non-obstructed kidney at one and two weeks after the release of UUO. This increase of the kidney weight was inhibited by PFD at two weeks. Five weeks after release of UUO, the weight of the obstructed kidney tended to decrease compared with the contralateral non-obstructed kidney. There was no significant difference between the weight of the obstructed kidney in two the

Table 1. Body weight (grams) of clearance study groups

	Weeks after release of UUO		
	1	2	5
Sham-operated	396 ± 3 (6)	ND	ND
Untreated control	300 ± 10 (3)	342 ± 7 (8)	430 ± 8 (6)
PFD treated	296 ± 4 (5)	329 ± 6 (7)	420 ± 9 (6)

Data are expressed the mean ± SE. Number of rats is in parentheses. ND is not determined in this study. Abbreviations are: UUO, unilateral ureteral obstruction; PFD, Pirfenidone.

Table 2. Kidney weight (grams) of clearance study groups

	Weeks after release of UUO					
	1		2		5	
	C	UUO	C	UUO	C	UUO
Sham-operated	1.21 ± 0.01	1.24 ± 0.01	ND		ND	
Untreated control	1.28 ± 0.14	1.36 ± 0.09	1.25 ± 0.03	1.43 ± 0.05[a]	1.64 ± 0.02	1.42 ± 0.14
PFD treated	1.14 ± 0.03	1.34 ± 0.07[a]	1.27 ± 0.04	1.28 ± 0.05	1.54 ± 0.11	1.45 ± 0.08

Data are expressed as the mean ± SE.
Abbreviations are: C, control kidney; UUO, unilateral obstructed kidney; ND, not determined in this study. Number of animals is same in Table 1.
[a] $P < 0.05$ compared to contralateral kidney

Table 3. Mean arterial pressure (mm Hg) of clearance study groups

	Weeks after release of UUO		
	1	2	5
Intact (sham)	104 ± 4 (6)	ND	ND
Control	107 ± 7 (3)	105 ± 3 (8)	99 ± 5 (6)
PFD	116 ± 6 (5)	111 ± 3 (7)	103 ± 4 (6)

Data are expressed the mean ± SE. Number of rats is in parentheses. ND is not determined in this study.

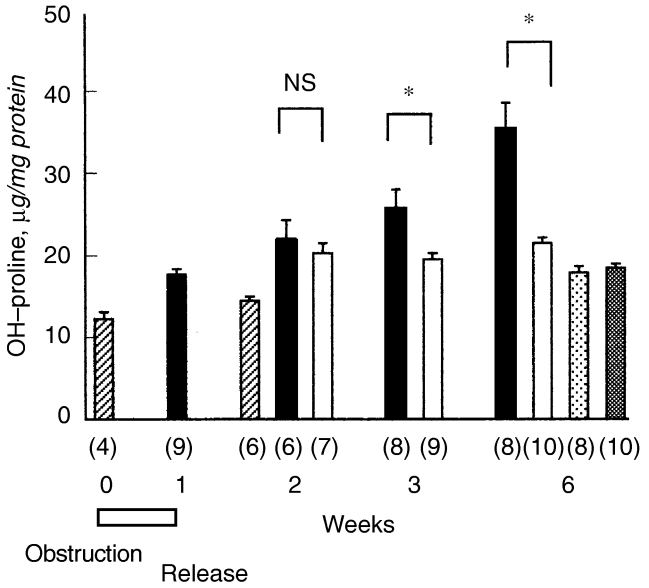


Fig. 5. Effect of PFD on cortical hydroxyproline content of obstructed kidney after the release of obstruction lasting one week. Data are represented as mean ± SE. The number of animals per group is shown in parentheses below each bar. Symbols are: (▨) sham-operated rat; (■) obstructed, untreated control rat; (□) obstructed, PFD treated rat; (⊡) contralateral, untreated control rat; (▩) contralateral, PFD treated rat. *$P < 0.05$ between with and without PFD treated groups in the same period; NS, not significant.

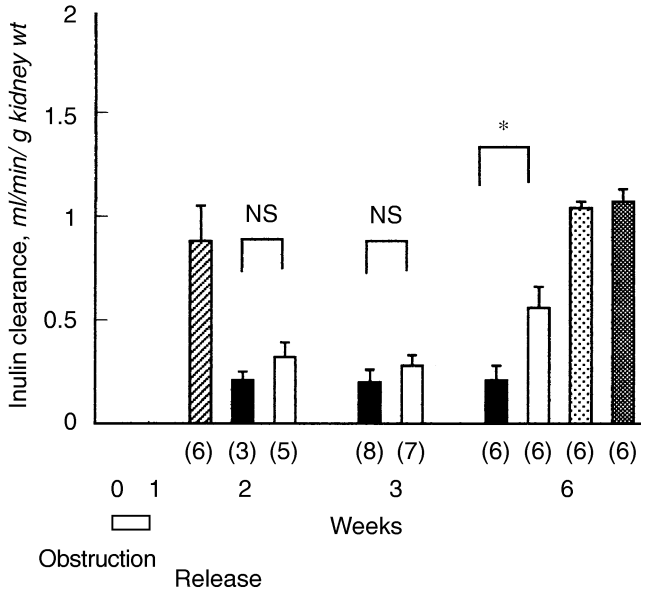


Fig. 6. Effect of PFD on inulin clearance of obstructed kidney after the release of obstruction lasting one week. Data are represented as mean ± SE. Number of animals per groups is shown in parenthesis below each bar. Symbols are: (▨) sham-operated rat; (■) obstructed, untreated control rat; (□) obstructed, PFD treated rat; (⊡) contralateral, untreated control rat; (▩) contralateral, PFD treated rat. *$P < 0.05$ between with and without PFD treated groups in same period; NS, not significant.

groups of UUO rats. There was no significant difference between blood pressure in the post-obstructed rats and in sham-operated rats for the periods studied (Table 3).

We examined the effect of PFD administration on collagen accumulation following release of obstruction after seven days of UUO (Fig. 5). In spite of the cancellation of the primary disorder by removal of the obstruction, a progressive increase of hydroxyproline content was still observed in the obstructed kidney. This increase was significantly reduced by PFD. Furthermore, this improvement remained significant 14 days after removal of the obstruction. In contrast with the untreated UUO group, no further increase of the hydroxyproline content was observed by five weeks in the PFD-treated group.

We next examined the effect of PFD on the renal dysfunction persisting after release of the obstruction after UUO. Inulin clearance was determined under anesthesia following administration of PFD by five weeks after release from UUO (Fig. 6). As compared with the contralateral non-obstructed kidney or sham-operated kidney, inulin clearance was markedly depressed in the post-obstructed kidney and remained at a low level throughout the observation period. No significant improvement was found after

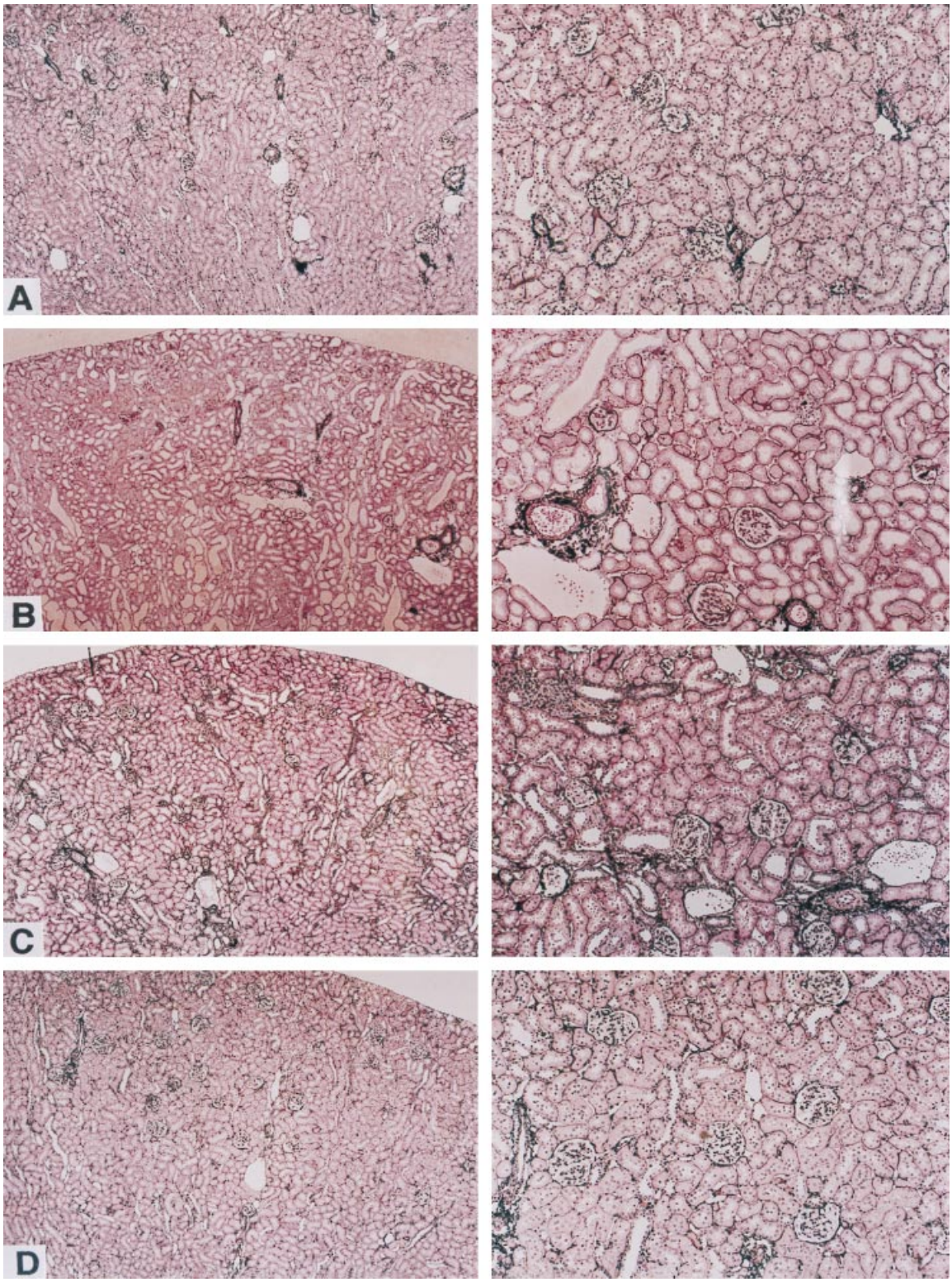


Fig. 7. Representative photomicrograph of PAM-stained sections from post-obstructed kidney five weeks after relief of obstruction lasting one week. (*A*) Normal cortex in contralateral non-obstructed kidney (×20, left; ×50, right). (*B*) Weakly positive staining was observed in the obstructed kidney after one week (×20, left; ×50, right). (*C*) A section of UUO showing a focal area of interstitial fibrosis and widened interstitial spaces in the kidney at five weeks after removal of the one week of UUO (×20, left; ×50, right). (*D*) Necrosis and sloughing of tubules were less common in the PFD-treated rats (×20, left; ×50, right).

two weeks following relief of obstruction in both the untreated and PFD-treated groups. However, administration of PFD for five weeks significantly improved inulin clearance. As compared with the untreated group, inulin clearance of the contralateral non-obstructed kidney was not different between the two groups with or without PFD

administration. These results indicate that PFD administration can attenuate impairment of renal function following removal of ureteral obstruction.

The histological appearance of the kidney at five weeks after removal of the seven-day ureteral obstruction showed variable and often extensive interstitial fibrosis. In comparison to the weakly and sparsely positive staining in the kidney cortex for the UUO after one week (Fig. 7B), strong and extensive interstitial staining persisted at week 5 after UUO release (Fig. 7C). There was widening of the interstitial spaces and some degree of hypercellularity. Prominent PAM positive staining was seen in the tubulointerstitial area. The glomeruli appeared to be generally well preserved, but sometimes were stained more readily in the glomerular area with periglomerular fibrosis. In mature glomeruli, the staining suggested sclerosis. By contrast, the PFD-treated animals showed striking attenuation of these histological changes with very little fibrosis in the interstitium (Fig. 7D). The histologic views of the obstructed kidney were quite different from those of the contralateral non-obstructed kidney (Fig. 7A).

DISCUSSION

Renal interstitial fibrosis displays the characteristic features of chronic obstructive nephropathy. Although the mechanisms responsible for UUO-induced kidney damage are not well understood, the fibrogenic process certainly leads to permanent loss of the normal integrity and function of the kidney. Clearly, relief of the obstruction is the best therapeutic option [30]. However, according to our data, prolonged obstruction induces progressive renal fibrosis with dysfunction which cannot be readily restored even with removal of the obstruction. Under these circumstances, pharmacotherapeutic intervention needs to be developed to reverse or halt the progression of the renal dysfunction that occurs as a consequence of the obstruction. Furthermore, the development of progressive interstitial fibrosis represents a final common pathway associated with a variety of kidney disorders that can lead to functional insufficiency [1, 2, 5]. Thus, the search for effective anti-fibrotic agents is of great importance not only for elucidating the mechanism of UUO-induced fibrosis, but also for alleviating the renal fibrosis seen under various conditions with CRF.

In the present study, we employed biochemical measures to quantitatively examine the processes of fibrogenesis in UUO. Our findings demonstrated that ureteral obstruction results in an accumulation of collagen in a time-dependent manner in the renal cortex of the obstructed kidney. The UUO-induced increase in the kidney hydroxyproline content was significantly inhibited by oral daily treatment with PFD for two to three weeks. Since accumulation of collagen in the kidney is the hallmark of renal fibrosis, a reduction in the collagen accumulation by PFD reflects its anti-fibrotic effect. Although the mechanisms for the ameliorating effect of PFD on UUO-induced fibrosis are not well understood, a decreased mRNA expression parallels a reduction in the accumulation of collagen, indicating that blocking renal fibrosis by PFD can prevent progressive renal disease, which in turn is associated with the prevention of an up-regulation of gene expression of collagen. As noted in this study, PFD can inhibit collagen accumulation when such accumulation is overexpressed. In contrast to other collagen-modulator substances, such as tumor necrosis factor-α (TNF-α) [31], interleukin (IL)-1 [32] and γ-interferon (γINF) [33], PFD does not alter the basal levels of collagen content. Therefore, PFD may be predominantly efficacious in a pathologic condition and may have the ability to normalize the over-expression of components that contribute to fibrosis.

The pathological role of TGF-β for extracellular matrix (ECM) accumulation in most forms of experimental nephritis has been well established [34]. Recent publications describing the signal that may trigger fibrosis have pointed out that TGF-β is probably a major cytokine responsible for the fibrotic reaction in normal tissue following various exogenous insults [35]. The sustained aberrant expression of renal TGF-β results in the pathologic accumulation of ECM material in interstitial compartments. This points to the challenge of determining which of the TGF-β-regulated events makes a significant contribution to the intrarenal fibrogenic process. We decided to examine the TGF-β gene expression. Our data indicate that inhibition of the UUO-induced increase of TGF-β mRNA in the kidney cortex occurs a few days earlier than the inhibition of increased collagen mRNA and is coincident with collagen accumulation. Thus, inhibition of collagen production by PFD may be mediated in part by the suppression of TGF-β. However, it remains unclear whether the decreased TGF-β expression is due to a direct effect of PFD and/or through a secondary mechanism accompanied by an anti-fibrotic action.

Monocyte-macrophage infiltration has been implicated as being pathogenetically important in cellular alterations contributing to the process of tubulointerstitial fibrosis. Indeed, such ED-1-positive cell infiltration was present in the obstructed kidney in an early phase after ureteral ligation [15]. Several cytokines secreted by infiltrating macrophage and T-lymphocytes act as chemoattractants and stimulate fibroblast proliferation. Such interstitial fibroblasts produce ECM protein and fibrosis. In addition, the macrophage—TGF-β axis is a plausible potential mechanism in the overproduction of ECM that may eventually cause interstitial fibrosis in the UUO model [36], since TGF-β is known to be a strong chemoattractant for monocytes [37]. Ishidoya et al [14, 15] found that enalapril, an angiotensin converting enzyme (ACE) inhibitor, significantly decreased the number of ED-1 positive monocytes infiltrating the interstitium and that this decreased the interstitial cellularity as well as contributed in part to the reducing interstitial fibrosis in the obstructed kidney. Although we have no evidence to indicate that there would be

a PFD effect on the inflammatory component to the response, the data in the present study showing that administration of PFD does not reverse the increased collagen accumulation in the acute phase of UUO offer little, if any, possibility for contribution from the adhesiveness of monocytes, which plays an essential role in mechanisms responsible for the anti-fibrotic effect of PFD. However, Diamond et al have shown that the ED-1 positive cell number does not reach maximum at seven days after UUO [38]. Therefore, to negate this possibility, further evidence is necessary to show that the effect of this treatment modality on chemoattractant levels, such as macrophage chemoattractant peptide-1 (MCP-1), intracellular adhesion molecule-1 (ICAM-1) and osteopontin, before excluding the effect of PFD on monocytic inflammation.

The other possible mechanism for the anti-fibrotic effect of PFD in the UUO model is to minimize the reactive oxygen species, because, as reported in a previous paper on a study using the bleomycin-induced lung fibrosis model in hamsters, PFD may prevent the initial bleomycin-induced production of reactive oxygen species or block their injurious effect by scavenging them [6]. The possibility is strengthened from the evidence that oxidant stress is thought to be a factor in experimental hydronephrosis [39].

Since the accumulation of ECM results from a balance between synthetic and degradative processes, a matrix-degrading system may also play an important role in regulating the turnover of collagen. To further define the possible contribution of the degrading system, we observed the effect on MMP-2, a matrix metalloproteinase, in UUO. In the present study, the MMP-2 gene was markedly induced by UUO. Therefore, it is less likely that depressed MMP production induces the accumulation of collagen. Furthermore, the anti-fibrotic effect of PFD does not appear to be due to a change in the MMP level, because the degree of its level was decreased by PFD administration. However, since in the present study we did not measure the activity of MMP *per se*, to establish the contribution of the matrix degrading system for the mechanism of the anti-fibrotic action of PFD, further studies are necessary to ascertain the enzyme activity of these proteinases.

Furthermore, our observation in this model of chronic interstitial injury after UUO seems paradoxical considering the change of the increment between collagen mRNA and hydroxyproline contents. Namely, the increase in type I and IV collagen mRNA levels peaked at one week post-UUO and declined when interstitial fibrosis was progressing. The data therefore lead us to believe that interstitial fibrosis may be due to decreased ECM degradation in the chronic phase. Indeed, Engelmyer et al [40] and Sharma et al [41] have demonstrated that there is impaired MMP activity by up-regulation of tissue inhibitors of MMP (TIMP) expression after UUO, suggesting that regulation of TIMP activity may contribute to the inception of the fibrogenic response. What remains to be determined is whether PFD can inhibit the activity of TIMP to provide the potential mechanism for the anti-fibrotic action.

The effects of obstruction on renal function depend upon the duration and completeness of the obstruction [42]. With complete obstruction, the degree to which the function recovers strongly depends upon the duration of obstruction. Few studies are available on the degree of recovery of renal function in humans [43], because of the difficulty for surgical release of the feasibility. Provoost and Molenaar [16] demonstrated that GFR, when measured in rats up to two weeks after the release of obstruction for varying periods of duration, recovered as much as 85% after removal of obstruction that had lasted one week. In contrast, Govan observed recovery after less than four days of UUO but no recovery after longer periods and concluded that repair did not occur [17]. These findings coupled with other data [44, 45] indicate that in rats, obstruction of two to three weeks is the maximum period that allows some recovery of renal function after removal of the obstruction. In the present study, functional recovery was much slower. Inulin clearance recovered to only 23% after the removal of UUO lasting one week against that of sham-operated rat but there was significant recovery to 63% with PFD treatment. In the present study, we demonstrated that reduction of UUO-induced collagen accumulation by PFD preceded the recovery from a decreased inulin clearance. These results clearly indicate that impairment of renal function associated with renal fibrosis can be substantially improved by the anti-fibrotic action of PFD. Our finding that progressive renal fibrosis with dysfunction cannot be readily restored in spite of removal of the obstruction agrees with the evidence from Diamond et al, who used this obstruction-release approach and found that it took four weeks for osteopontin mRNA expression to normalize after releasing the obstruction [38].

The morphologic observations of marked reduction in the fibrotic lesions in PFD-treated UUO rats as compared to the controls are encouraging. The reduced lesions in the PFD group implies amelioration of UUO-induced injury and suggests the possible therapeutic use of PFD in obstructive nephropathy.

In conclusion, PFD prevented UUO-induced interstitial fibrosis, and this was accompanied by significantly improved renal function. These experiments also demonstrate that PFD can prevent the development of irreversible progression of CRF in rats. Our results strongly support the concept that PFD may offer a new and exciting possibility for retarding the progression of human CRF.

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