J Korean Med Sci 2003; 18: 527-33
ISSN 1011-8934

Pirfenidone Suppressed the Development of Glomerulosclerosis in the FGS/Kist mouse

Pirfenidone (PFD) is a newly developed anti-fibrotic agent. We evaluated the effect of PFD for the prevention of renal fibrosis using a spontaneous progressive glomerulosclerosis animal model, FGS/Kist mice. Male and female FGS/Kist mice were fed a diet containing 0.5% PFD or the same control diet (CD) without PFD, for 1, 2, or 3-month periods. Body weight was monitored for the general effect of PFD on the mice. Proteinuria and glomerular filtration rate (GFR) were evaluated for renal function. The sclerosis index was examined for the morphological changes. There were no significant changes in body weight between the PFD and control groups in both sexes. Proteinuria levels were low in all the PFD groups compared to the corresponding CD groups. The sclerosis scores were also reduced in both sexes of the 3-month PFD groups ($p<0.05$), and glomerular filtration rates were increased in both sexes of the 3-month PFD groups compared to the CD groups. The treatment of PFD for 1 or 2-month periods did not have statistic significances but the treatment for 3 months had statistic significances in sclerosis and GFR compared to CD groups. These results suggested that long-term administration of PFD suppressed the progression of glomerulosclerosis and improved renal function of the FGS/Kist mice.

Key Words : *Pirfenidone; Fibrosis; Disease Models, Animal; Glomerulosclerosis, Focal; Glomerular Filtration Rate*

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Received : 28 February 2003
Accepted : 15 May 2003

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*This research was supported by the Yeungnam University Research Grants in 1998 and by the Yeungnam University Hospital Research Grant in 1997.

INTRODUCTION

Fibrosis is a pathologic process of tissue remodeling accompanied with an accumulation of extracellular matrix (ECM) proteins. Several organs and tissues are affected by fibrosis such as kidney, lung, liver, heart, vessel, peritoneum or skin. The etiology of fibrosis is a tissue injury like inflammation, radiation, wound etc., but still many cases are idiopathic. Even though the pathogenesis of fibrosis is not fully understood yet, it is caused by imbalance between the accumulation and the degradation of ECM. Several factors, such as TGF-β, connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), reactive oxygen intermediates, or matrix metalloproteinase and their inhibitors are involved in the regulation of synthesis and degradation of ECM with complex network according to the type of tissues or organs (1-8). At the present time, fibrotic diseases of various organs in human are treated with corticosteroids and cytotoxic drugs that have serious systemic adverse effects (9, 10).

Pirfenidone (PFD) is a recently developed drug and it is intensively studied as a therapeutic agent for fibrosis of different organs (11-23). There are several reports to examine the ability of PFD on renal fibrosis. The researchers used diverse animal models such as 5/6 nephrectomy rats, unilateral ureteral obstructed rats or rabbit, streptozotocin-diabetic rat or vanadate-induced kidney fibrosis rats for the evaluation of the activity of PFD against fibrosis (16-19). The FGS/Kist mouse develops a progressive glomerulosclerosis genetically and revealed characteristic morphology of focal glomerulosclerosis (24-28). Therefore, it is regarded as a suitable animal model for the study of human spontaneous progressive glomerulosclerosis (24-28). Consequently, we employed FGS/Kist mouse to determine if PFD is effective in the preventing the spontaneous progressive glomerulosclerosis. The data presented in this paper indicated that long-term intake of PFD reduced ongoing progress of renal fibrosis and improve renal function.

MATERIALS AND METHODS

Animals

FGS/Kist mice were offered by KIST (Korea Institute of Science and Technology). The 6-weeks old male and female

FGS/Kist mice were treated with PFD (Marnac Inc. Dallas, TX, U.S.A.) for 1, 2, and 3- month periods with four heads mice in each group. Powdered food mixed with PFD was freely fed to the treatment groups, and the same food but without PFD was fed to the control groups. The concentration of the PFD was adjusted to 0.5% (w/w) in order to offer the dose at 500 mg/kg/day. All the animals were maintained under the barrier system of an air-conditioned room at 22±2°C, 55±5% relative humidity, and 12 hrs L/D cycle, and had free access to tap water.

Measurement of Proteinuria and body weight

Proteinuria of each mouse was checked once a week with Uriscan strips (Yeong-dong Pharmaceutical Corp., Seoul, Korea) and the body weight was also checked. Proteinuria was scored as 1 point for "+", 1.5 point for "+~++", and 2 point for "++" so on, and the mean proteinuria of each group was calculated. The median value of "+" is 30 mg/dL, "++" is 100 mg/dL, "+++" is 300 mg/dL and "++++" is 1,000 mg/dL.

Measurement of Glomerular filtration rate (GFR)

GFR was assessed by modified single injection of ^{99m}Tc–DTPA method which was previously reported (29, 30). ^{99m}Tc–DTPA (Du Pont-Nuclear, Boston, MA, U.S.A.) was administered to the peritoneum of the mice at the end date of the treatment. Blood was collected by cardiac puncture 60 min after the injection of radioisotope. Radioactivity in the plasma was counted by γ-counter (Cobra II, Packard, U.S.A.) and the GFR was calculated with the following formula:

$$C=(V/t)\times \log_n (P_0/P_t)$$

where C is the GFR (mL/min) and V (mL) is the distribution volume of isotope calculated from body weight (29). P_0 is the injected amount of radioactivity (cpm/mL) and P_t is the amount of radioactivity in the plasma (cpm/mL) taken at 60 min after the injection.

Morphological investigations

Kidneys of the mice were removed immediately after taking the blood sample for GFR test. The left kidney was cut into two pieces by sagittal section and fixed in 2.5% formalin-Histochoice (Amresco, U.S.A.) for light microscopic examination. The right kidney was snap-frozen in liquid nitrozen and kept in -80°C for in situ hybridization. The formalin fixed tissues were embedded in paraffin and sectioned at 3 μm, and then stained with periodic acid-Schiff (PAS) for histological examination by light microscopy. The severity of the glomerulosclerosis was estimated by the modified semi-quantitative scoring system proposed by Raij et al. (31). Briefly, 100 glomeruli in each specimen were examined by microscope at a magnification of ×400, and the severity of the lesion was graded from 0 to 2 according to the percentage of glomerular involvement. Thus, grade 0 represented normal glomerular; grade 1 lesion indicated mild or moderate segmental hyalinosis and/or sclerosis involving less than 50% of the glomerular tuft; grade 2, diffuse glomerulosclerosis with more than 50% of the tuft involved. The sclerosis score in each animal was expressed as a mean of all scores obtained.

$$\text{Sclerosis score} = \frac{\text{No. of } <50\% \text{ sclerosis glomeruli} \times 1 + \text{No. of } >50\% \text{ sclerosis glomeruli} \times 2}{100 \text{ glomeruli}}$$

Statistical Analysis

The statistical significance was studied by Wilcoxon rank sum test. Data are presented as mean±SEM of four mice. Statistical significance was set at $p<0.05$.

RESULTS

The Changes of Body Weight

The mean body weight of each period PFD group was compared with that of each period of same sex CD group at the beginning and the end of the treatment (Table 1). The last body weights of male CD, male PFD, female CD, and female PFD groups were 25.9, 25.7, 20.8, and 20.4 g in the 1-month period groups, respectively. They were 32.5, 30.1. 25.9, and 25.5 g in the 2-month period groups and 29.1, 27.4, 25.7, and 23.6 g in the 3-month period groups, respectively. There were no significant differences in the mean body weight between the PFD groups and same sex CD groups ($p>0.05$) except for the 3-month period female groups ($p<0.05$).

PFD Decreased Proteinuria

The mean proteinuria levels was checked as one of the markers of renal damage and compared at the beginning and at the

Table 1. Effects of Pirfenidone on body weight of FGS/Kist mice

Tx* Periods		Male		Female	
		Before Tx	After Tx	Before Tx	After Tx
1 Month	CD	20.5±1.4	25.9±1.9	16.7±0.8	20.7±1.4
	PFD	23.1±1.7	25.7±1.6	16.6±0.9	20.4±1.3
2 Month	CD	24.1±1.1	32.5±2.0	19.1±1.7	25.9±2.7
	PFD	23.1±1.4	30.1±4.5	19.8±1.0	25.5±0.2
3 Month	CD	19.3±1.2	29.1±4.5	16.0±0.6	25.7±1.6
	PFD	18.2±0.5	27.4±2.3	18.4±0.5	23.6±1.5*

Mean body weights of each group were monitored at the beginning and the end of treatment of pirfenidone. Each value represents mean±SEM of four mice. Rate of gain weight of 3 month female PFD group was significantly lower than those of 3 month female CD group (*$p<0.05$), Wilcoxon rank sum test. *Tx: treatment.

end of the experiment in each group (Table 2). In the 1-month groups, the proteinuria scores increased at the end of the treatment 62.5, 22.2, 63.6 and 47.3% in male CD, male PFD, female CD, and female PFD groups, respectively when compared to before the treatment. They are also increased 45.4, 29.4, 33.3, and 42.8% in the 2-month and 45, 31.5, 33.3, and 7.69% in the 3-month male CD, male PFD, female CD, and female PFD groups, respectively. The proteinuria increase was less in PFD groups compared with each period of the CD groups (except the 2-month female groups) and were statistically significant the 1-month male and 3-month female groups ($p<0.05$).

Effects of PFD on Renal Function

Renal function was evaluated by the GFR. In the 1-month groups, the GFR of the PFD group was lower than the CD group in both sexes, and the GFR was similar in all 2-month groups (Fig. 1). The GFR was 23.8 and 23.5% higher in the 3-month PFD male and female groups compared to the 3-month CD male and female groups, respectively, and was statistically signiticant in the 3-month female groups ($p<0.05$). The GFR was increased in all of the 2-month period groups (0.39-0.43 mL/min/g) compared to the 1-month period groups (0.28-0.39 mL/min/g) but decreased in all of the 3-month period groups (0.23-0.40 mL/min/g) compared to the 2-month period groups.

Table 2. Effects of PFD on proteinuria of FGS/Kist mice

Tx* Periods		Male		Female	
		Before Tx	After Tx	Before Tx	After Tx
1 Month	CD	1.1±0.3	3.0±0	1.0±0	2.8±0.5
	PFD	1.8±0.3	2.5±0.6*	1.3±0.5	2.4±0.5
2 Month	CD	1.5±0.6	2.8±0.5	1.3±0.5	1.9±0.6
	PFD	1.5±0.6	2.1±0.6	1.0±0	1.8±0.5
3 Month	CD	1.4±0.5	2.5±0.6	1.8±0.3	2.6±0.5
	PFD	1.6±0.5	2.4±0.8	1.5±0.6	1.6±0.3*

Mean proteinuria scores of each group were monitored at the beginning and the end of treatment of Pirfenidone. Each value represents mean±SEM of four mice. The increasing rate of mean proteinuria was significantly lower in 1-month male and 3-month female PFD groups than 1-month male and 3-month female CD groups, respectively (*$p<0.05$), Wilcoxon rank sum test. Tx: treatment.

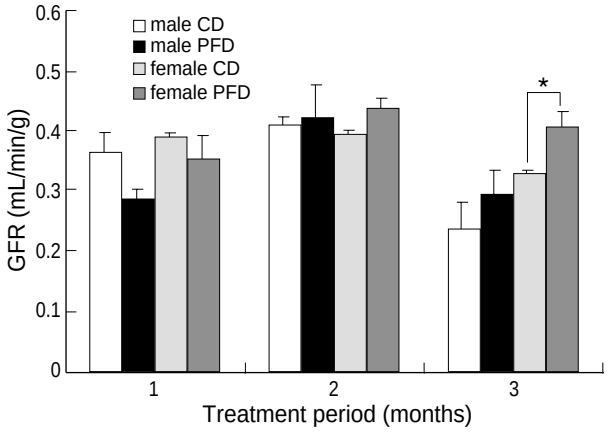

Fig. 1. Effect of PFD on GFR. Glomerular filtration rates were measured as described in Materials and Methods. Each value represents mean±SEM of four mice. GFR of 3-month female PFD group was significantly higher ($p<0.05$) than 3-month female CD group, Wilcoxon signed rank test.

PFD Suppressed Development of Sclerosis

The sclerosis scores were very low (between 0.03-0.06) in all of the 1-month groups and there were no significant differences between PFD groups and control groups in both genders (Fig. 2). The characteristic glomerular change of FGS/Kist mice was sclerosis; segmental collapse of glomerular capillary tufts and luminal obliteration with matrix protein. It started from very small area of glomerulus of 1-month group and gradually involved in whole glomerulus, which was well demonstrated in 3-month male CD group (Fig. 3A, C). The number of sclerotic glomeruli was also increased by age. The tubular atrophy and interstitial fibrosis as well as interstitial lymphocytic infiltration were also associated with similar degree of glomerular sclerosis in CD groups (Fig. 3A). However, PFD protected the development of glomerulosclerosis and tubulointerstitial fibrosis significantly (Fig. 3B, D). The sclerosis score increased according to get old in male CD groups, such as 0.03, 0.41, and 1.04 in 1, 2, and 3-month groups, respectively. But it was only 0.04, 0.21, 0.23 in corresponding male PFD groups and there is a significant difference between the 3-month CD and PFD male groups ($p<0.05$) (Fig. 2). There was no significant increase of sclerosis in the 2-month female groups (0.05 and 0.09 in CD and PFD groups, respectively), when compared to the 1- month groups (0.05 and 0.06 in CD and PFD groups, respectively). In the 3-month female groups,

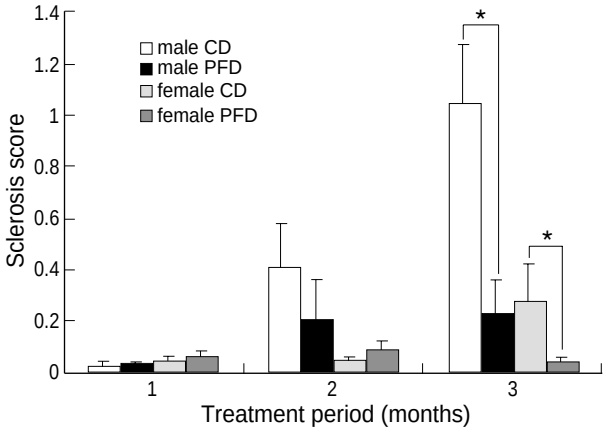

Fig. 2. Effect of PFD on sclerosis. Sclerosis scores were measured as described in Materials and Methods. Each value represents mean±SEM of four mice. Sclerosis scores of 3-month month male and female PFD groups were significantly lower than corresponding 3-month CD groups ($p<0.05$), Wilcoxon rank sum test.

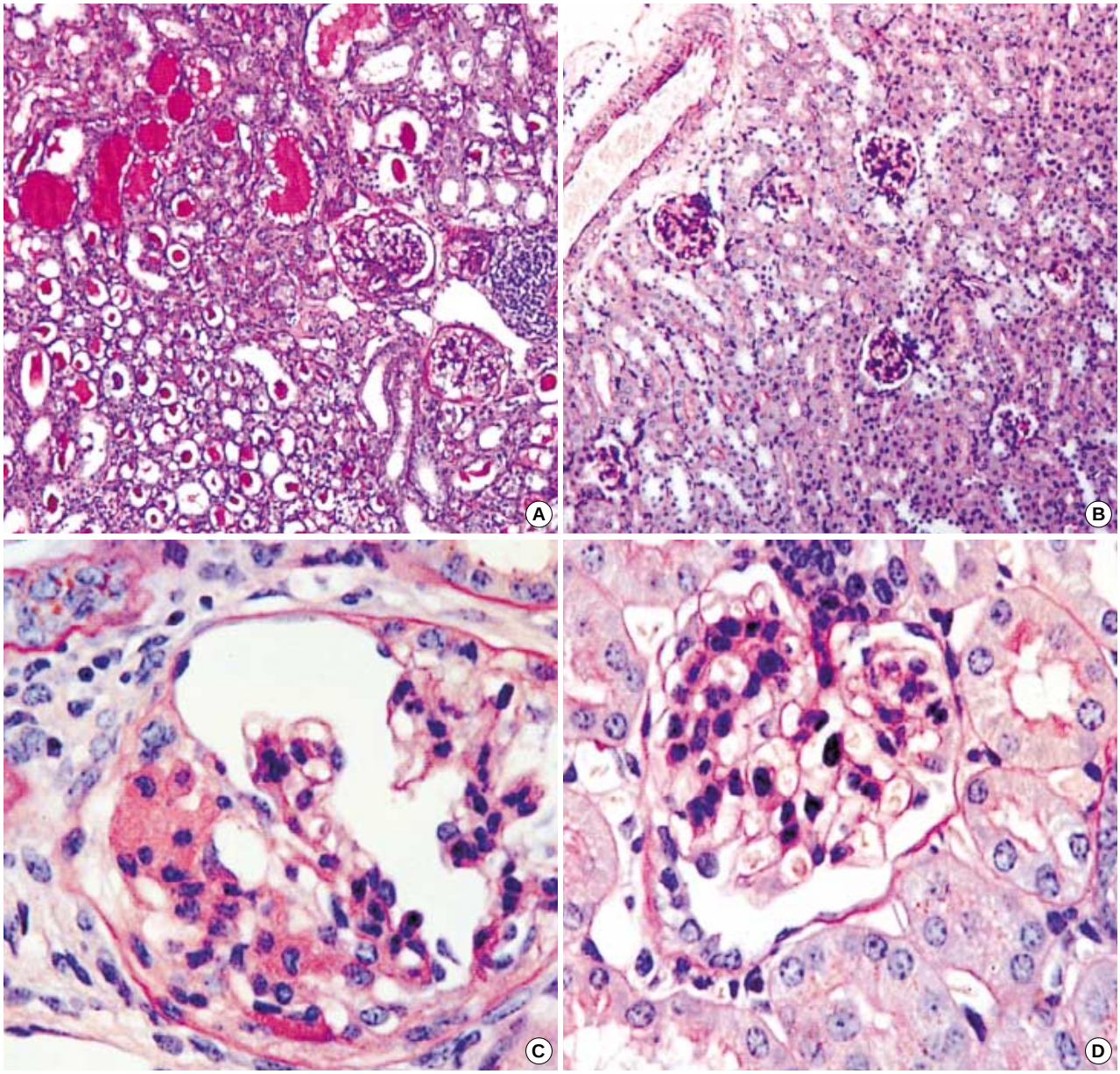

Fig. 3. Morphologic changes of kidney tissue. (A, C) FGS/Kist mouse kidney of 3-month male CD group shows glomerulosclerosis and tubulointerstitial injury pattern, segmental collapse of glomerular capillary tufts and luminal obliteration with matrix protein (A, PAS stain, ×40, C, ×400). (B, D) These changes are significantly ameriolated in 3-month male PFD group (B, ×40, D, ×400).

the sclerosis score increased to 0.28 in the CD group but it was still 0.04 in the PFD group ($p<0.05$) (Fig. 2). Therefore, PFD reduced sclerosis 78% in 3-month male and 84% in 3-month female groups compared to corresponding CD groups ($p<0.05$).

DISCUSSION

Renal fibrosis is a complication of kidney injury and can contribute to renal failure regardless of its preceding disease. It may develop not only as a result of nephrectomy, chronic infection, obstruction of ureter, malignant hypertension, severe diabetic condition, or intoxication of heavy metals but also idiopathic. Several kinds of renal fibrosis animal models are used for the evaluation of efficacy and mechanism of drugs or to elucidate the mechanism of pathogenesis. Common methods to induce renal fibrosis are 5/6 nephrectomy, unilateral ureteral obstruction, administration of streptozotocin, injection of vanadate, or administraion of puromycin (16-19, 32, 33). All of the above renal fibrosis models represent special situations such as removed, obstructive, diabetic, inflamma-

tory or intoxicated kidney.

The FGS/Kist mouse, which originated from the FGS/Nga mouse, develops a spontaneous progressive glomerulosclerosis (24-28). The FGS/Nga mouse was established from the F_5 offspring of a cross-breeding of CBA/Nga and RFM/Nga mice (24, 25). It is known that the two pairs of autosomal recessive genes are involved in the disease of this animal (24). In the previous reports, the FGS/Kist strain showed progression of proteinuria and focal glomerulosclerosis with the aging (26, 27). Those findings resemble the focal glomerulosclerosis of human and this animal is considered as a suitable animal model for the idiopathic glomerulosclerosis (24-28).

Pirfenidone (PFD) is a broad spectrum anti-fibrotic agent developed recently. There are many reports about the effectiveness and mechanism of the PFD against several kinds of fibrotic disease such as lung fibrosis, renal fibrosis, liver cirrhosis, sclerosing peritonitis etc. (11-23). We employed FGS/Kist mice to evaluate the effectiveness of PFD on the spontaneous glomerulosclerosis.

As a parameter for general condition, we checked body weight. The proteinuria levels were monitored and glomerular filtration rates were evaluated for the renal function. During the administration of PFD, there were no significant differences on body weight between the CD and the PFD treatment groups in each treatment periods except in the 3-month female groups (Table 1). This result suggested that the administration of PFD for 3 months might not cause significant toxicity to FGS/Kist mice. In the beginning of the treatment (6 weeks-old mice), the proteinuria levels were similar (scores were between 1-1.75) in all groups (Table 2). The level of proteinuria had increased in all groups at the end of the experiment but there was a tendency for less increases in the PFD groups (score 1.63-2.5) compared to the corresponding CD groups (score 1.88-3.0). The effectiveness of PFD against proteinuria, before and after the treatment, was statistically valid ($p<0.05$) in the 3-months months male and female groups (Table 2).

Renal function was evaluated by GFR. The GRF was increased in the 2-month groups compared to the 1-month groups and then decreased in 3-month groups when compared to the 2-months groups (Fig. 1). It was considered that the increase of the GFR in the 2-months groups might be due to growth of the mice without severe disease, and the decrease of the GFR in the 3-months groups was because of the progression of the sclerosis. The GFR of the 2-months groups was similar in all groups (0.41, 0.42, 0.39, and 0.43 mL/min/g in the male CD, the male PFD, the female CD, and the female PFD groups, respectively). The GFR of the 3-months groups decreased 42.1, 30.3, 16.5, and 6.67% in the male CD, male PFD, female CD, and female PFD groups, respectively, when compared with corresponding 2-months groups. The severity of fibrosis was evaluated by sclerosis score. In all 1-month groups, there was no significant sclerosis even though they had proteinuria (Fig. 2, Table 2). The proteinuria probably preceded the morphological change in the FGS/Kist mouse. Instead of CD group, in which the sclerosis scores had increased abruptly, the sclerosis scores did not increase significantly in the 2 and 3-months PFD male groups (Fig. 2). In female CD groups, there was no significant sclerosis until the 2-months groups, and then the score increased in the 3-months groups, but there was no significant sclerosis in the PFD female groups of all periods. Not only the glomerulosclerosis, tubulointerstitial fibrosis also developed in FGS/Kist mice, but the treatment of PFD also improved the tubulointerstitial fibrosis (Fig. 3). Therefore, the treatment of PFD for 3 months improved renal function and suppressed the development of sclerosis.

Accumulation of extracellular matrix (ECM) is the hallmark in glomerulosclerosis and subsequently leads to end-stage renal disease. It occurs as a result of an imbalance between synthesis and degradation of matrix proteins. Therefore, balancing the rate of synthesis and degradation of ECM is one of the targets for the treatment of glomerulosclerosis. TGF-β is known as a most important cytokine in the pathogenesis of fibrosis. TGF-β regulates the turnover of ECM via several different pathways. For example, TGF-β stimulates the synthesis of ECM, protease inhibitors, integrins, or reduces the synthesis of ECM-degrading proteases (2). On the other hand, major physiologic degradation of ECM is regulated by the matrix metalloproteinases (MMPs) such as collagenases, gelatinases, stromelysins, and membrane-type matrix metalloproteinases. Stromelysin-1 (MMP-3), one of the MMPs, degrades ECM and has a broad substrate specificity, including IV-collagen, laminin, fibronectin, and proteoglycans (34). The catalytic activity of MMPs is regulated at multiple levels including transcription, secretion, activation, and inhibition. Inhibition of MMPs activity is accomplished by the members of the tissue inhibitors of the metalloproteinase (TIMP) family such as TIMP-1, -2, -3 and -4. TIMP-1, -2 and -3 are present in the kidney (6, 7). TIMP-1 is a glycoprotein produced by virtually all mesenchymal tissues including intrinsic glomerular cells, fibroblasts and inflammatory cells such as macrophages (6). TIMP-1 inactivates stromelysin, interstitial collagenase and the latent gelatinase. The importance of MMP-3 and TIMP-1 in the turnover of ECM has been reported in several models or diseases of progressive renal scarring (3).

In the present study, we also observed mRNA expression of TGF-β, MMP-3 and TIMP-1 by northern blot to investigate the mechanism of anti-fibrotic action of PFD. TGF-β was decreased in PFD group compared to the CD and 2-month male group showed statistical significance (data not shown). TIMP-1 was all decreased in all male PFD groups but not significant statistically (data not shown). However, MMP-3 was detected only in 3 months groups and decreased in male PFD group (data not shown). The induction of MMP-3 at 3-month group was considered as a physiologic balance for the increase of TIMP-1 and fibrosis. There was a tendency that TGF-β and TIMP-1 mRNA expressions were decreased in 2-month and 3-month male PFD groups compared to the corresponding male CD groups, but they were increased in female. There-

fore, the effects of PFD on the mRNA expression of TGF-β, TIMP-1 and MMP-3 showed some discrepancies between both sexes. The disease of FGS/Kist is related with two autosomal recessive genes, even though we do not know yet what kind of genes they are (24). When we considered the following data such as FGS/Kist mice are inbred mice (24), they have immune complexes and retroviral envelop antigen in their kidney (25), the morphology of renal disease of FGS/Kist mice resemble the pathology of the kidney disease of human immunodeficiency acquired disease (personal communication), there is a possibility that the disease of FGS/Kist mice might be related with endogenous retroviruses (ERV). Some diseases such as rheumatoid arthritis, systemic lupus erythematosus, which are known to related with ERV, have some gender preferences (35-37). Therefore, if the disease of FGS/Kist mice was related with ERV, there could be some differences in the development of the disease in male and female. It is obvious that PFD improves renal function and suppresses sclerosis in both sexes of FGS/Kist mice, but we could not find the consistent molecular mechanism of PFD in both sexes of FGS/Kist mice in the transcriptional level. However, the mechanism of the fibrosis is a very complicate network in vivo and involved several molecules in transcriptional, and post-transcriptional level, we could not explain the mechanism of PFD in this study. Therefore, further studies are needed to confirm the mechanism of PFD in the prevention of glomerulosclerosis.

From the above data, we conclude that 1) the proteinuria developed in young FGS/Kist mice and preceded fibrotic changes, 2) the severity of the disease depends on both age and gender (males showed a greater severity of disease), 3) long-term (3 months) administration of pirfenidone improved renal function and suppressed sclerosis in the FGS/Kist mice.

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