PIRFENIDONE INHIBITS DIMETHYLNITROSAMINE-INDUCED HEPATIC FIBROSIS IN RATS

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INTRODUCTION

Pirfenidone (PFD), 5-methyl-1-phenyl-2(1H)-pyridone, is a compound that can prevent and even reverse extracellular matrix (ECM) accumulation in several organs, as shown by experimental studies on pulmonary fibrosis,1 peritoneal sclerosis2 and renal sclerosis.3,4 In patients with pulmonary fibrosis, PFD not only restores pulmonary function, but also improves survival rate.1 Pirfenidone has also been reported to inhibit fibroblast proliferation in vitro in response to a number of growth factors.3 Although these pharmacological actions of PFD as an antifibrotic agent have been established, little is known about the effects of PFD on hepatic fibrosis.

Hepatic fibrosis occurs in patients with chronic liver disease (e.g. persistent viral hepatitis, alcohol overload and autoimmune liver disease) as a consequence of severe liver damage. Regardless of the causes, hepatic fibrosis involves an abnormal accumulation of ECM components, particularly collagens.6–8 Hepatic stellate cells (HSC; also referred to as Ito cells, fat-storing cells or lipocytes) are non-parenchymal liver cells residing in the perisinusoidal space of Disse.8–10 In studies of cultured HSC,7,11 experimental fibrosis 12 and human liver disease,13 HSC have been found to be the major cellular source of ECM.4,14–16

In the present study, we evaluated the effect of PFD on experimental hepatic fibrosis induced by dimethyl nitrosamine (DMN) in rats. Pirfenidone suppressed the induction of hepatic fibrosis and reduced hydroxyproline levels and expression of mRNA for type I collagen in a dose-dependent manner. These results demonstrate that PFD inhibits DMN-induced hepatic fibrosis by preventing the accumulation of collagen produced by HSC and suggest that PFD may be potentially useful in the prevention of the development of hepatic fibrosis.

Key words: dimethyl nitrosamine, hepatic fibrosis, hepatic stellate cells, hydroxyproline, pirfenidone, type I collagen.

METHODS

Animal study

Male Wistar king A rats (200–250 g; Laboratory of Animal Experimentation, Kyushu University, Kyuhsu, Japan) were maintained on a basal pelleted diet and water ad libitum in a room under normal laboratory lighting conditions. Dimethyl nitrosamine (Sigma Chemical Co., St Louis, MO, USA) was dissolved in saline (final concentration 1%) and 10 mg/kg per day DMN was injected intraperitoneally (i.p.) on the first 3 days of each week for 4 weeks, as described previously.17,18 Pirfenidone (Shionogi, Tokyo, Japan) was dissolved in dimethyl sulphoxide (DMSO; final concentration 20%) and 500 mg/kg PFD was given orally every day for 4 weeks starting on the day of the first injection of DMN. Twenty rats were randomized into four experimental groups, as follows: (i) S-D, saline i.p. and oral administration of DMSO; (ii) S-PFD, saline i.p. and oral administration of PFD; (iii) DMN-D, DMN i.p. and oral administration of DMSO; and (iv) DMN-PFD, DMN i.p. and oral administration of PFD. Rats were killed at the end of the 4th week and livers were excised. A blood sample was obtained immediately before rats were killed. Protocols for these studies were reviewed by the Committee on the Ethics of Animal Experimentation in...
the Faculty of Medicine, Kyushu University and were performed according to the control of the Guidelines for Animal Experimentation of the Faculty of Medicine, Kyushu University and The Law (No. 105) and Notification (No. 6) of the Japanese Government.

**Histopathology**

For light microscopic examination, liver specimens were fixed overnight in phosphate-buffered formaldehyde, embedded in paraffin and stained with Masson’s trichrome stain. The extent of fibrosis was quantified in histological sections using a computer-assisted morphometric analyser (MacScope; Mitani Corporation, Fukui, Japan), as reported previously. Briefly, a field containing a portal vein of approximately 100 μm in diameter in its centre was selected under a magnification of ×100. Digitalized images of the field and an adjacent eight fields of the same size were captured for computer analysis by a digital camera (Fujix HC-1000; Fujith, Tokyo, Japan). Collagen (stained light green by Masson trichrome) was delineated in the images and its area was measured by the analyser. The extent of fibrosis in each field was expressed as the area of collagen as a percentage of the total area of the field. Eliminating the maximum and minimum values among the nine fields examined, the average of the remaining seven fields was calculated for assessment of the degree of fibrosis in each case.

**Measurement of tissue hydroxyproline content**

For measurement of hydroxyproline, a portion of each liver was homogenized in 10 volumes (mL/g) of saline at 4°C using a Polytron PCU-2 homogenizer (Kinematica, Lucerne, Switzerland). Quantitative determination of hepatic hydroxyproline levels was performed using a high-performance liquid chromatographic (HPLC) system, as described previously (SRL, Tachikawa, Japan). Hydroxyproline levels were analysed separately for each liver twice.

**Semi-quantitative reverse transcription–polymerase chain reaction assay for type I collagen mRNA**

Extraction of total RNA and a semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) assay were performed, as reported previously. Briefly, the PCR primers were constructed on the basis of the polymerase chain reaction (RT-PCR) assay were performed, as reported previously. The competitor DNA sequence for GAPDH PCR was generated by PCR using the following composite primer, 5'-CTG ACT GGA AGA GCC GAG AG-3' (sense) and 5'-TGA GTT TGG GTT GTT GTT CT-3' (antisense)) and the rat glycerol aldehyde 3-phosphate dehydrogenase (GAPDH) gene (GenBank accession number Z78279; 5'-GCC AAG TTC AA T GGC ACA GT = G TGG AGT = 5'). *P < 0.05 compared with DMN-D.

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| S-D, saline i.p. and oral administration of dimethylsulfoxide (DMSO); S-PFD, saline i.p. and oral administration of pirfenidone (PFD); DMN-D, dimethyl-nitrosamine (DMN) i.p. and oral administration of DMSO; DMN-PFD, DMN i.p. and oral administration of PFD; ALT, alanine aminotransferase.

**Northern blot analysis for TGF-β mRNA**

Northern blot analysis was performed as described previously. Total RNA (10 μg) was size fractionated on a 1.0% agarose gel in 3-morpholino propane sulfonic acid (MOPS) buffer (20 mmol/L), pH 7.0, and transferred to a Hybond-N nylon membrane (Amershams Life Science, Buckinghamshire, UK). This membrane was probed by an alkaline phosphatase enzyme-labelled rat TGF-β2 or rat GAPDH using the Gene Images Alkphos Direct labelling and detection system (Amershams) according to the manufacturer’s instruction. The detection was performed using the CDP-Star chemiluminescent detection system (Amershams).

**Aminotransferase assay**

l-Alanine aminotransferase (ALT) activity in the serum was assayed by standard spectrophotometric methods using commercial test reagents (GPT-OA test; Wako, Osaka, Japan).

**Cell isolation and culture**

The HSC were isolated from the liver of male Wistar rats by sequential in situ perfusion with collagenase and digestion with pronase, followed by centrifugation in a double-layered (17%/11.5%) Metrizamide solution (Sigma Chemical), as described previously. The HSC were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 20% fetal calf serum (FCS), 50 U/mL penicillin and 50 μg/mL streptomycin. Experiments described in the present study were performed on cells between the second and fourth serial passages.

**Type I collagen assay**

Cultured HSC were incubated in serum-free medium in the presence or absence of PFD for 48 h. Pirfenidone was dissolved in DMSO and added to cultures at less than 0.5% of the total volume of the media. Type I collagen was determined in culture media by an enzyme-linked immunosorbent assay (ELISA), as described previously. Anti-rat type I collagen antibody (LSL, Osaka, Japan) was used as first antibody and peroxidase-conjugated goat antibody against rabbit IgG (Organon Teknika, Durham, NC, USA) was used as the second antibody. Rat tail tendon collagen type I (Advance Biofactures, Lymbrook, NY, USA) was used as the standard. Results are expressed as μg collagen/mL, which was determined by a fluorometric assay according to the method of Brunk et al.. In all experiments, the medium was supplemented with 0.5% DMSO as a control for the possible effects of

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Table 1 | Effect of pirfenidone administration on the gain in bodyweight, liver weight and serum alanine aminotransferase levels

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DMSO on the cells. Cell viability was greater than 95% in all experiments, as determined by Trypan blue exclusion. Cell viability was greater than 90% as also determined by the 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (MTT Cell Growth Assay Kit; Chemicon International, Temecula, CA, USA).

**Statistical analysis**

All results are shown as the mean±SD. Comparisons were made using one-way ANOVA followed by Scheffé’s test or the Mann–Whitney U-test.

**RESULTS**

**PFD prevents bodyweight loss induced by DMN**

The effect of oral administration of PFD on bodyweight and liver weight of rats with and without i.p. injection of DMN is shown in Table 1. Treatment with DMN caused a significant decrease in bodyweight and liver weight (DMN-D group) compared with control animals (S-D group). Oral PFD essentially prevented this DMN-induced loss of bodyweight (DMN-D group; \( P < 0.05 \)) and tended to suppress the loss in liver weight (\( P = 0.11 \)). However, there were no significant differences in serum ALT levels between the DMN-D and DMN-PFD groups at the end of the 4th week.

The S-PFD group also received PFD (500 mg/kg) daily for 4 weeks, but without DMN. No mortality or major adverse effects were observed in this group. There were no significant differences in bodyweight, liver weight or serum ALT levels between the control group (S-D) and the S-PFD group and liver histology in the latter group appeared completely normal. These findings suggest that PFD has no major side effects in vivo. However, these preliminary results do not entirely exclude potential side effects that may appear following longer periods of administration of PFD.

**Histopathology and hepatic hydroxyproline content**

Intraperitoneal administration of DMN for 4 weeks resulted in a uniform fine granulation of the surface of the liver. Microscopic analysis revealed cirrhotic-like structural patterns in the DMN-D group; that is, fibrous connective tissue components in Glisson’s sheath, pseudolobule formations and formation of fibrotic septa and thickened reticulin fibres joining central areas (Fig. 1a). In contrast, livers of rats that received DMN and PFD for 4 weeks (DMN-PFD...
Pirfenidone inhibits hepatic fibrosis

Fig. 3  Semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of α1 (I) collagen mRNA expression. Total RNA was extracted from the liver of (a) untreated rats (S-D) or rats treated with either (b) dimethylnitrosamine (DMN) alone (DMN-D) or (c) DMN plus pirfenidone (DMN-PFD). Samples were analysed by competitive RT-PCR using primer pairs specific for α1 (I) collagen after adjustment to ensure that equal amounts of cDNA were present in each PCR (based on the results of the competitive PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH)). The series of competitive PCR for α1 (I) collagen were performed using the same amount of cDNA in each PCR with the following amounts of the α1 (I) collagen competitor: 20 fg (lane 1); 2 fg (lane 2); 0.2 fg (lane 3); 0.02 fg (lane 4). The DMN-induced increase in α1 (I) collagen mRNA was inhibited by PFD. Each result is representative of three independent experiments.

Fig. 4  Northern blot analysis of transforming growth factor (TGF)-β mRNA expression. Pirfenidone (PFD) suppressed the dimethylnitrosamine (DMN)-induced increase in TGF-β expression in the liver. The filter was rehybridized with a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as a control. The DMN-induced increase in TGF-β mRNA was partially inhibited by PFD.

Fig. 5  Effect of PFD on type I collagen accumulation in vitro showing type I collagen levels in culture media in the presence or absence of pirfenidone (PFD). Type I collagen in culture media was analysed as described in Methods. Pirfenidone inhibited type I collagen accumulation in a concentration-dependent manner, down to a minimum inhibitory concentration of 5 mmol/L. Data are the mean ± SD. *P < 0.05 compared with the values for hepatic stellate cells in the absence of PFD.

PFD inhibits type I collagen and TGF-β mRNA expression

To assess the level of inhibition of type I collagen mRNA transcription by PFD, we examined the level of α1 (I) collagen mRNA in DMN-treated liver by semiquantitative RT-PCR. In the control group (S-D group), even in the presence of only 0.02 fg competitor, a major competitor-specific band was detected in the competitive RT-PCR for α1 (I) collagen (Fig. 3a). In contrast, in the DMN-D group, DMN treatment enhanced the transcription of the α1 (I) collagen gene (in the absence of PFD) and, even in the presence of 2 fg competitor, resulted in a band representing the α1 (I) collagen product (Fig. 3b). Treatment with PFD resulted in a more intense competitor band; in the presence of 0.02 fg competitor, the α1 (I) collagen PCR product was present at an intensity equal to that of the competitor (Fig. 4c). These results demonstrate that treatment with PFD suppresses DMN-induced α1 (I) collagen mRNA expression in the liver.

Next, we examined the effects of PFD on TGF-β mRNA expression by northern blot analysis, because TGF-β has been reported to be a major cytokine responsible for the fibrotic reaction in the liver. Treatment with DMN increased TGF-β expression in the liver; however, PFD partially suppressed the DMN-induced increase in expression of TGF-β (Fig. 4).

Type I collagen accumulation in vitro

To assess the effect of PFD on ECM production by HSC, type I collagen concentrations in culture media were analysed after 2 days incubation in the presence or absence of PFD. The Trypan blue
exclusion test and the MTT assay showed that more than 90% of cells survived these treatments. Treatment of cells with increasing concentrations of PFD led to a dose-dependent suppression of collagen accumulation and 5 mmol/L PFD reduced the accumulation of collagen to approximately 50% (Fig. 5).

**DISCUSSION**

Hepatic fibrosis (cirrhosis) is the final common pathway for all forms of chronic liver disease (e.g. persistent viral hepatitis, alcohol overload and autoimmune liver disease), progressing to end-stage hepatic failure. Although cirrhosis is an important cause of long-lasting morbidity and death, newly developed drugs used to treat chronic liver diseases cannot successfully prevent the progression of hepatic fibrosis and cirrhosis.18-20 In the present study, we evaluated the antifibrotic agent PFD for its ability to block hepatic fibrosis in a rat model induced by DMN. A significant ameliorating effect of PFD on hepatic fibrosis was demonstrated: PFD significantly suppressed not only the induction of hepatic fibrosis by 40%, as evaluated by image analysis, but also the increase in the tissue hydroxyproline content. Because accumulation of collagen in the liver is the primary hallmark of hepatic fibrosis, these results reflected the antifibrotic effect of PFD.

Pirfenidone has been reported to prevent ECM accumulation in other organs, as shown by experimental studies on pulmonary fibrosis,21 peritoneal sclerosis22 and renal sclerosis.23,24 The mechanism by which this new compound offered significant protection against these fibroses is not fully understood. In the case of hepatic fibrosis, PFD suppressed upregulated mRNA expression of the α1 (I) collagen gene in the liver and led to a dose-dependent suppression of collagen accumulation in vitro in primary HSC cultures. The HSC are activated in hepatic fibrosis and represent the major cellular source of ECM. Taken together, these findings suggest that one of the major targets for the action of PFD is the HSC and that PFD suppresses hepatic fibrosis, at least in part, by preventing the activation of HSC.

In this DMN model, a low dose of DMN initially causes diffuse haemorrhage necrosis, leading to infiltration of mononuclear cells.25,26 Monocyte–macrophage infiltration has been implicated as being pathogenetically important in cellular alterations contributing to the process of hepatic fibrosis, because several cytokines, such as TGF-β, secreted by infiltrating cells trigger the activation of HSC.27 In particular, TGF-β has been reported to be a major cytokine responsible for the fibrotic reaction in the liver.27,28 In the present study, PFD suppressed increased TGF-β mRNA expression in the DMN-treated liver. Pirfenidone has also been reported to suppress increased TGF-β mRNA expression in the postobstructed kidney29 and the bleomycin-treated lung,30 suggesting that these antifibrotic effects of PFD may be mediated, in part, by the suppression of TGF-β. In in vitro studies, we found that PFD inhibited collagen production in a dose-dependent manner. Although it would be of interest to assess whether PFD suppresses TGF-β-induced collagen synthesis, we found that TGF-β did not, in fact, induce an increase in collagen production by the activated HSC that were used in the present study (data not shown). In agreement with our findings, Dooley et al.31 have reported that fully activated HSC (after day 7 from isolation) produce collagen maximally but no longer respond to TGF-β stimulation. The effects of PFD on HSC activation, including TGF-β-induced collagen production, remain to be examined using unactivated HSC.

Another possible mechanism for the antifibrotic effect of PFD in hepatic fibrosis may be to reduce the production or action of reactive oxygen species (ROS), because a previous report has suggested that PFD may prevent the bleomycin-induced production of ROS or scavenge ROS after production.1 Recent reports also suggest that lipid peroxidation may be a link between liver tissue injury and fibrosis.32,33 In the present study, there was no significant difference in serum ALT levels between PFD-treated (DMN-PFD) and -untreated groups (DMN-D). A single administration of DMN caused a rapid increase in serum ALT levels that peaked at approximately day 3 and, thereafter, returned to normal.34 Because we collected blood 5 days after the last DMN administration, ALT levels had possibly already returned to near normal levels, which resulted in a lack of significant differences between the two groups. It is possible that serum ALT levels in DMN-D may be much higher than those in DMN-PFD at their peak because of scavenging of ROS by PFD.3 Further in vivo and in vitro studies, including the use of different liver cell types, such as hepatocytes, Kupffer cells and sinusoidal endothelial cells, will be required to determine whether agents may be therapeutically effective for the treatment of hepatic injury and fibrosis.

In conclusion, the use of PFD during hepatic injury induced by DMN was shown to be very effective in blocking fibrogenesis without any apparent adverse side effects. Therefore, the present study provides a foundation for the development of novel therapies for hepatic fibrogenesis.

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**REFERENCES**

11. Friedman SL. Cellular sources of collagen and regulation of collagen
12. Martinez-Hernandez A. The hepatic extracellular matrix. II. Electron
immunohistochemical studies in rats with CCl₄-induced cirrhosis. *Lab.
Invest.* 1985; **53**:166–86.
types involved in collagen and fibronectin production in normal and
14. Friedman SL, Roll FJ, Boyle J, Bissell DM. Hepatic lipocytes: The
Sci. USA* 1985; **82**:8681–5.
15. Arenson DM, Friedman SL, Bissell DM. Formation of extracellular
matrix in normal rat liver: Lipocytes as a major sources of proteoglycan.
*Gastroenterology* 1988; **95**:441–7.
16. Maher JJ, Friedman SL, Roll FJ, Bissell DM. Immunolocalization of
laminin in normal rat liver and biosynthesis of laminin by hepatic
F. A morphological study of the early stages of hepatic fibrosis induced
by low doses of dimethylnitrosamine in the rat. *J. Hepatol.* 1987; **5**:
174–81.
18. Jezequel AM, Mancini R, Rinaldesi ML et al. Dimethylnitrosamine-
induced cirrhosis. Evidence for an immunological mechanism. *J.
Hepatol.* 1989; **9**:42–52.
19. Tada S, Iwamoto H, Nakamuta M et al. Selective ROCK inhibitor,
Y27632, inhibits dimethylnitrosamine-induced hepatic fibrosis in rats.
*J. Hepatol.* 2001 (in press).
fluorometric detection system for liquid chromatographic analysis of
amino and amino acids using o-phthalaldehyde/N-acetyl-L-cysteine
Expression of collagen alpha1 (I) mRNA variants during tooth and bone
ization of rat and human glyceraldehyde-3-phosphate dehydrogenase
cDNAs: Genomic complexity and molecular evolution of the gene.
protein in the regulation of actin cytoskeleton in hepatic stellate cells.
*J. Hepatol.* 1999; **31**:91–9.
24. Tsuji T, Okada F, Yamaguchi K, Nakamuta T. Molecular cloning of the
large subunit of transforming growth factor type beta masking protein
Sci. USA* 1990; **87**:8835–9.
A p160ROCK-specific inhibitor, Y-27632, modulates rat hepatic
stellate cell activation. *J. Hepatol.* 2000; **32**:762–70.
26. Brunk KC, Jones KC, James TW. Assay for nanogram quantities of DNA
27. Matsuoka M, Tsukamoto H. Stimulation of hepatic lipocyte collagen
production by Kupffer cell-derived transforming factor beta: Implication
for a pathogenetic role in alcoholic liver fibrogenesis. *Hepatology* 1990;
**11**:599–605.
28. Tsukamoto H. Cytokine regulation of hepatic stellate cells in liver
30. Iyer SN, Gurujeyalakshimi G, Giri N. Effects of pirfenidone on trans-
dermatation of rat hepatic stellate cells to myofibroblasts. *J. Pharmaco-
Modulation of transforming growth factor β response and signaling
during transdifferentiation of rat hepatic stellate cells to myofibroblasts.
32. Hougum K, Filip M, Witztum JL, Chojkier M. Malondialdehyde and
4-hydroxynonenal protein adducts in plasma and liver of rats with iron
Stimulation of collagen α1 (I) gene expression is associated with lipid
peroxidation in hepatocellular injury. A link to tissue fibrosis? *Hepatol-
34. Shiba M, Shimizu I, Yasuda M, Li K, Ito S. Expression of type I
and type III collagens during course of dimethylnitrosamine-induced
hepatic fibrosis. *Liver* 1998; **18**:196–204.