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Pirfenidone suppresses tumor necrosis factor-α, enhances interleukin-10 and protects mice from endotoxic shock

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Abstract

A new experimental drug, pirfenidone (5-methyl-1-phenyl-1*H*-pyridine-one; S-7701), has been reported to have beneficial effects for the treatment of certain fibrotic diseases. We investigated the anti-inflammatory properties in murine endotoxic shock to determine the pharmacological characteristics. The present study describes the prophylactic effect, cytokine regulatory profiles and therapeutic effect of pirfenidone in murine endotoxic shock, which was induced in mice using an intraperitoneal (i.p.) injection of lipopolysaccharide and D-galactosamine. First, we examined the prophylactic effect and cytokine regulatory profiles. A single oral administration of pirfenidone prior to lipopolysaccharide/D-galactosamine challenge inhibited the production of circulating tumor necrosis factor-α (TNF-α), interleukin-12 and interferon-γ, markedly enhanced that of interleukin-10, and offered protection from subsequent lethal symptoms in a dose-dependent manner. Second, we examined the therapeutic effect. A single oral administration of pirfenidone 1, 2, 3, 4 and 5 h post lipopolysaccharide/D-galactosamine challenge provided protection against lethal shock in a time-dependent manner. At the histopathological level, apoptotic positive cells were found to be suppressed in the liver. The transforming growth factor (TGF)-β1 level was markedly elevated in the liver of lipopolysaccharide/D-galactosamine-challenged mice, suppressed in pirfenidone-treated mice. These findings may offer an alternative for both protective and therapeutical treatment of several human acute or chronic inflammatory diseases by pirfenidone.

Keywords: Endotoxic shock; Inflammatory cytokine; TNF-α (tumor necrosis factor-α); Interleukin-10; Apoptosis; Fibrosis; TGF-β (transformation growth factor-β)

1. Introduction

Most cases of human septic shock, which are systemic responses to severe bacterial infections resulting in high mortality, are caused by Gram-negative bacterial endotoxins (Gilbert, 1960; Morrison and Ryan, 1987; Dannaer et al., 1991). In experimental animals, lipopolysaccharide challenge leads to pathophysiological changes similar to the human septic shock syndrome. The toxic effects of endotoxin are exerted through the generation of endogenous pro-inflammatory cytokines. High levels of circulating tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-6, interleukin-10, interleukin-12 and interferon-γ were reported during endotoxemia (van Deuren et al., 1992). Among these cytokines, inflammatory cytokines TNF-α, interleukin-12 and interferon-γ are the key factors in the development of septic disease (Heinzel et al., 1994; Ozmen et al., 1994; Wysocka et al., 1995). Neutralizing or soluble receptors against TNF-α, interleukin-12 or interferon-γ as well as their deficiencies have been reported to suppress the induction of septic shock reactions (Beutler et al., 1985; Tracey et al., 1987; Pfeffer et al., 1993; Beutler and Cerami, 1989; Lesslauer et al., 1991; Ashkenazi et al., 1991; Angehrn et al., 1993; Magram et al., 1996a,b; Car et al., 1994; Tsutsui et al., 1997), and interleukin-10 is known as an anti-inflammatory cytokine to decrease circulating TNF-α and offer protection against endotoxic shock (Fiorentino et al., 1991; Howard et al., 1993). These findings suggest that regulators of these cytokines can be candidates for anti-inflammatory agents.

Pirfenidone is a newly developed anti-fibrotic agent with the chemical structure of 5-methyl-1-phenyl-1*H*-pyridine-2-one shown in Fig. 1. Pirfenidone can safely arrest progression of existing fibrotic lesions (Iyer et al., 1995; Margolin and Lefkowitz, 1994), reduce or remove excessive fibrotic lesions and prevent the development of fibrotic lesions (Margolin and Lefkowitz, 1994). In relation to the mechanism of the anti-fibrotic action, pirfenidone has been shown

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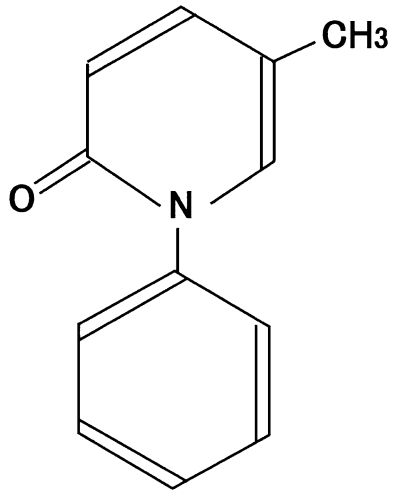


Fig. 1. Chemical structure of pirfenidone (5-methyl-1-phenyl-1*H*-pyridine-2-one: S-7701).

to modify cytokine regulatory actions, inhibit fibroblast proliferation and collagen matrix synthesis. However, the detailed mechanism has remained unknown.

In mice, i.p. injection of pirfenidone has been shown to block endotoxin-induced shock, as well as the endotoxin-induced marked elevation of circulating TNF-α (Cain et al., 1998). Doses efficacious against the murine endotoxic shock model correspond well to those of experimental fibrotic disease models, which is more than 200 mg/kg. Based on these findings, we hypothesized that both the functional efficacies, anti-fibrotic and anti-inflammatory actions, might be caused by the same mechanism of this agent. We therefore tried to study the anti-inflammatory actions of pirfenidone to evaluate the functional mechanism by using the murine acute endotoxic shock model.

We present data showing the unique anti-inflammatory actions of pirfenidone, with full protection of murine endotoxin-induced lethal shock, by modifying the production of inflammatory cytokines (inhibition of TNF-α, interleukin-12, interferon-γ and enhancement of interleukin-10), therapeutic protection and suppression of subsequent hepatic apoptosis and tissue transforming growth factor (TGF)-β1 elevation in hemorrhagic necrotic liver failure in vivo.

2. Materials and methods

2.1. Animals and reagents

C57BL/6 mice (8 to 11 weeks, female) were purchased from Charles River Japan (Osaka). Pirfenidone (S-7701) was purchased from ACIC Inc. (Canada). Lipopolysaccharide from *Escherichia coli* strain O55:B5 prepared by Boivin's method was obtained from Difco Laboratories (Detroit, MI, USA). Murine-recombinant interferon-γ was prepared at Shionogi (Osaka, Japan). Lysis buffer was purchased from New England BioLabs (Beverly, MA). D-Galactosamine–HCl was from Tokyo Chemicals (Tokyo). Carboxymethylcellulose (CMC) was purchased from Nacalai Tesque (Kyoto, Japan).

2.2. Experimentally induced endotoxic shock and protocols

C57BL/6 mice received oral (p.o.) administrations of pirfenidone at the times indicated for each experiment. Murine endotoxic shock was induced by intraperitoneal (i.p.) injection with lipopolysaccharide (50 μg/kg) and D-galactosamine (250 mg/kg). The survival rate was monitored over the next 3 or 5 days. For determination of the serum cytokine levels, each mouse was sacrificed and blood samples were obtained. The samples were centrifuged and the serum was collected and stored at −80 °C until use for cytokine determination. Murine-recombinant interferon-γ was introduced into mice by i.p. injection. For tissue observation, livers were obtained from individual mice at the times indicated and stored in buffered 10% formalin until use. Sterilized phosphate-buffered saline (PBS) was used as a solvent for lipopolysaccharide and D-galactosamine, and control animals received 200 μl of PBS. Pirfenidone was suspended in 0.5% CMC solution. All experiments using animals were followed according to Animal Care and Use Committee of Shionogi (Shionogi).

2.3. ELISAs

Enzyme-linked immunosorbent assay (ELISA) kits for murine interleukin-1β, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-6 and interleukin-12 (p40 and p70) were purchased from Amersham Pharmacia. The ELISA kit for murine TNF-α, interleukin-10, interleukin-12 (total), interferon-γ and human TGF-β1 was purchased from Genzyme Techne (Minneapolis, MN). The ELISA kit for murine interleukin-18 was purchased from MBL (Nagoya, Japan). All ELISA assays were performed as indicated by the manufacturer's instructions. Detection limits were 23.4 pg/ml for TNF-α, 15.6 pg/ml for interleukin-10, 20 pg/ml for interleukin-12, 9.4 pg/ml for interferon-γ and 31.2 pg/ml for TGF-β1.

2.4. Histopathology and immunohistochemistry

For the microscopic observations under light microscope, two histological sections were prepared from the fixed tissues. One was stained with hematoxylin–eosin (HE) and the second by the Tunel method. For Tunel staining, the Apop-Tag in situ Apoptosis Detection Kit-Peroxidase (Oncoa) was used as described by the manufacturer.

2.5. TGF-β1 quantitation in the liver tissue using ELISA kit

For determination of TGF-β1 level in the liver tissue, TGF-β1 protein content in the tissue homogenate was determined by ELISA. One hundred milligrams of each

liver was suspended in 1 ml of cell lysis buffer (BioLabs) on ice and vigorously homogenized by polytron homogenizer (PT 10-35). The homogenate was centrifuged at 3000 rpm for 10 min (Kubota 5700) and the supernatant was obtained. The supernatant was acid-treated and neutralized according to the manufacturer's protocol of human TGF-β1 ELISA kit (Genzyme Techne), and sampled into ELISA kit. Murine TGF-β1 quantitation using human TGF-β1 ELISA kit was already reported (Maltman et al., 1996).

2.6. Statistical analyses

All values are given as means ± S.D. Serum cytokine levels in different experimental groups were analyzed for statistical significance using Dunnett's *t*-test and Student's *t*-test. A value of $P < 0.05$ was considered to represent a significant difference.

3. Results

3.1. Prophylactic effect of pirfenidone on lipopolysaccharide-induced lethal shock in mice

The lipopolysaccharide/D-galactosamine-induced murine endotoxic shock model (Galanos et al., 1979; Lehmann et al., 1987; Decker and Keppler, 1974) was used to assess the anti-inflammatory effect of pirfenidone. First, the prophylactic effect was studied by a single administration of pirfenidone 15 min prior to lipopolysaccharide/D-galactosamine challenge in several dosages (100, 300 or 500 mg/kg, p.o.). Fig. 2 shows the results. After lipopolysaccharide/D-galactosamine challenge, mice began to die at 5–6 h and most mice died within 24 h in the vehicle-treated group. Pretreatment with pirfenidone (500 mg/kg, p.o.) provided full protection from lethal shock (8/8 mice survived). Pirfenidone administration (100 mg/kg, p.o.) still offered half protection from the lethal shock (4/8 mice survived). Pirfenidone shows the prophylactic effect on lipopolysaccharide/D-galactosamine-induced lethal shock in mice in a dose-dependent manner. This effect was not observed with p.o. administration of pirfenidone 24 or 6 h prior to lipopolysaccharide/D-galactosamine challenge, and a partial effect was observed 3 h before lipopolysaccharide/D-galactosamine challenge (data not shown).

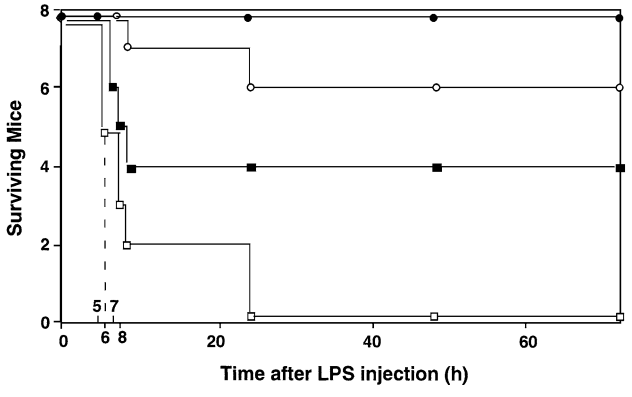


Fig. 2. Effects of pirfenidone pretreatment on lipopolysaccharide/D-galactosamine-induced lethal shock. A single administration of pirfenidone (0, 100, 300, 500 mg/kg, p.o.) was given to mice ($n = 8$/group), followed by i.p. injection of D-galactosamine/lipopolysaccharide 15 min later. Pirfenidone was suspended in 0.5% CMC vehicle. The mice began to die 5–6 h after lipopolysaccharide/D-galactosamine challenge, with most dying during the first 12 h. Surviving mice were monitored for 72 h. Open squares, vehicle treated; closed squares, 100 mg/kg of pirfenidone treated; open circles, 300 mg/kg of pirfenidone-treated; closed circles, treated with 500 mg/kg of pirfenidone.

3.2. In vivo inhibition of serum TNF-α, interleukin-12, interferon-γ and enhancement of interleukin-10 levels by pirfenidone

To determine the protective effect of pirfenidone in lipopolysaccharide/D-galactosamine-challenged mice, the serum levels of inflammatory cytokines, TNF-α, interleukin-10, interleukin-12, interferon-γ, interleukin-1β and interleukin-6 were studied by ELISA. Pirfenidone (500 mg/kg, p.o.) was administered to mice, followed by lipopolysaccharide/D-galactosamine challenge 5 min later, and each serum cytokine level was determined at the indicated times. As shown in Fig. 3, pirfenidone significantly suppressed TNF-α, interleukin-12 and interferon-γ but markedly enhanced the interleukin-10 level (TNF-α, 97% suppression in 75 min; interleukin-12, 84% suppression in 3 h; interferon-γ, 91% suppression in 4.5 h; interleukin-10, 41.5-fold enhancement in 3 h after lipopolysaccharide/D-galactosamine challenge, respectively). Interleukin-1β and interleukin-6 were suppressed less than 50% under the same conditions (data not shown). Next, the dose-dependent effect on each cytokine was studied. Pirfenidone suppressed TNF-α, interleukin-12 and interferon-γ, but enhanced interleukin-10 (Fig. 4) in a dose-dependent manner. Interleukin-10 level was markedly enhanced approximately by 27.7-fold when pirfenidone (500 mg/kg) was administered.

3.3. Therapeutic effect of pirfenidone on lipopolysaccharide-induced lethal shock in mice

The therapeutic effect of pirfenidone was studied after lipopolysaccharide/D-galactosamine challenge following a single administration of pirfenidone (500 mg/kg, p.o.). Because lipopolysaccharide/D-galactosamine-challenged mice began to die in 5–6 h, pirfenidone (500 mg/kg, p.o.) was administered to mice at 1, 2, 3, 4 or 5 h post lipopolysaccharide/D-galactosamine challenge, and the surviving mice were monitored (Fig. 5). In the group treated with pirfenidone 1, 2, 3 or 4 h post lipopolysaccharide/D-galactosamine challenge, 5/5, 4/5, 4/5 or 3/5 mice survived in 120 h, respectively. In the group treated by pirfenidone 5 h later, surviving mice were observed (1/5). This survival rate was dependent on the administration time.

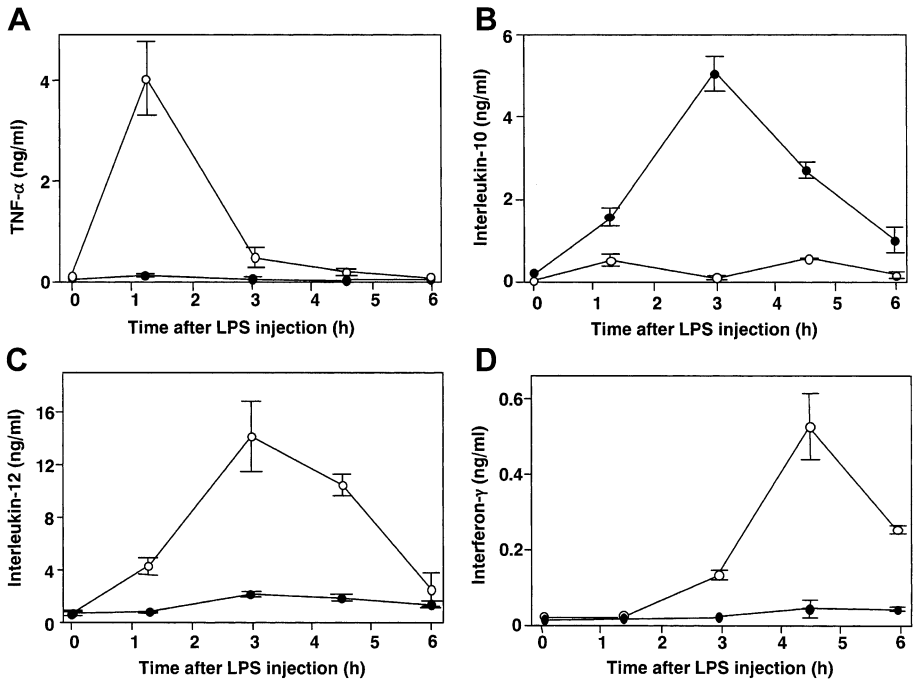


Fig. 3. Regulatory effects of pirfenidone on systemic secretion of circulating cytokines, TNF-α, interleukin-12, interferon-γ and interleukin-10 in lipopolysaccharide/D-galactosamine-injected mice. Mice were given or not given pirfenidone (500 mg/kg, p.o.) and injected with lipopolysaccharide/D-galactosamine 5 min later ($n = 3$/group). Control mice were orally administered 200 μl of 0.5% CMC vehicle. Serum was collected from each mouse at the indicated times after lipopolysaccharide/D-galactosamine challenge. Cytokine levels of mice untreated (open circles) or pirfenidone treated (closed circles) were determined by ELISA. (A) TNF-α, (B) interleukin-10, (C) interleukin-12 and (D) interferon-γ.

3.4. Suppression of serum interferon-γ level in the therapeutic protection

In the therapeutic process, the involvement of interleukin-12, interferon-γ and interleukin-10 was studied. The serum levels of interleukin-12, interferon-γ and interleukin-10 was monitored in mice treated by pirfenidone 3 h post lipopolysaccharide/D-galactosamine challenge by ELISA. Although pirfenidone showed no effect on circulating interleukin-12 and interleukin-10 levels in 4.5 h post lipopolysaccharide/D-galactosamine challenge, only interferon-γ was suppressed (data not shown). Suppression of interferon-γ was possibly involved at least in the therapeutic protective effect by pirfenidone.

3.5. In vivo injection of murine-recombinant interferon-γ into lipopolysaccharide/D-galactosamine sensitized mouse pre- or posttreated by pirfenidone

To determine the possibility of involvement of interferon-γ in the therapeutic effect, direct injection of murine-recombinant interferon-γ into mouse, which was pre- or posttreated by pirfenidone, was done. In the preliminary study, 1 μg/mouse of recombinant interferon-γ was injected (i.p.) into mice, serum interferon-γ concentration was detected at approximately 2 ng/ml after 1 h of the injection (data not shown). This level is higher than the level induced by lipopolysaccharide/D-galactosamine injection (Fig. 4D, interferon-γ level was approximately 0.6 ng/ml). From the preliminary studies, 1 μg/ml of recombinant interferon-γ was injected (i.p.) into mice pre- or posttreated by pirfenidone. The results are shown in Table 1. In group I, sensitized only by lipopolysaccharide/D-galactosamine, all mice died. In group II, pretreated by pirfenidone, all mice survived. In group III, posttreated by pirfenidone 3 h after lipopolysaccharide/D-galactosamine sensitization, almost all mice (lethality: 10%) survived. In group IV, pretreated by pirfenidone and interferon-γ injected 4 h after lipopolysaccharide/D-galactosamine sensitization, all mice survived. In group V, posttreated by pirfenidone and interferon-γ injected 4 h after lipopolysaccharide/D-galactosamine sensitization, half of the mice (lethality: 50%) died. In group IV, TNF-α may be suppressed and interferon-γ may be circulated. In group V, both TNF-α and interferon-γ may be circulated. From these results, both TNF-α and interferon-γ were considered to be involved in the lethality of mice. However, the posttreatment effect of pirfenidone cannot be explained by interferon-γ suppression only because half of the mice who survived were observed in group V.

3.6. Lipopolysaccharide-induced expression of apoptotic positive cells and suppression by pirfenidone in murine lipopolysaccharide-induced liver failure

As hepatocyte apoptosis and subsequent hemorrhagic necrosis in the liver were reported to be the main effector

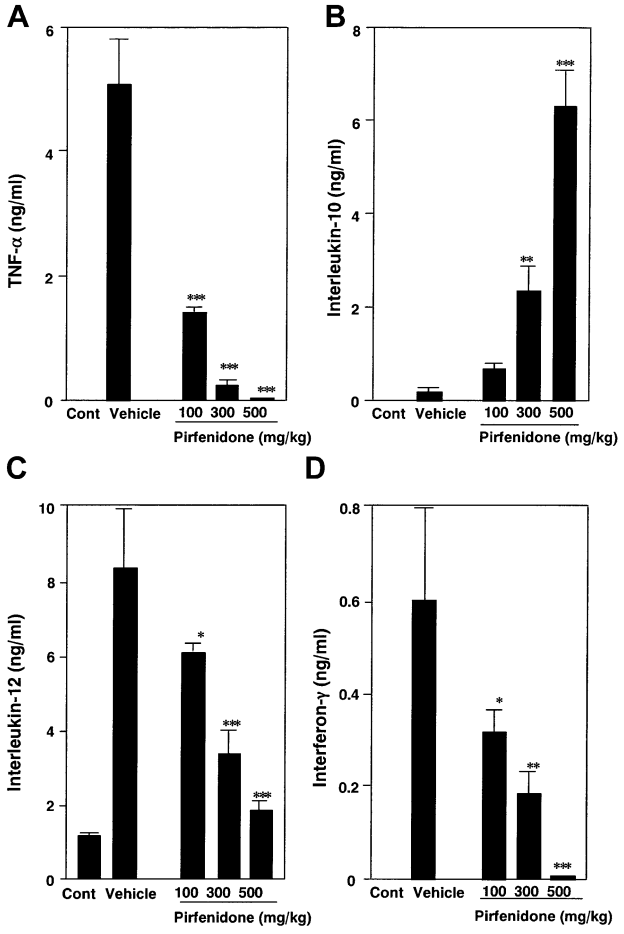


Fig. 4. Dose-dependent effect of pirfenidone on serum TNF-α, interleukin-10, interleukin-12 and interferon-γ levels after lipopolysaccharide/D-galactosamine-challenge. Mice were each orally administered a dosage of pirfenidone and challenged 5 min later with lipopolysaccharide/D-galactosamine (i.p.). (A) Sera were taken 75 min after lipopolysaccharide/D-galactosamine challenge and analyzed for the production of TNF-α ($n=4$/group) by ELISA. (B) Sera were taken 3 h after lipopolysaccharide/D-galactosamine challenge for interleukin-10 ($n=3$/group). (C, D) Sera were taken 5 h after lipopolysaccharide/D-galactosamine challenge and analyzed for interleukin-12 ($n=4$/group) or interferon-γ ($n=4$/group) by ELISA. Each column represents the mean ± S.D. $*P<0.01$, $**P<0.001$, $***P<0.0001$ (Dunnett's t-test) significantly different as compared to vehicle-treated group.

events in murine lipopolysaccharide/D-galactosamine-induced endotoxemia (Galanos et al., 1979; Levy et al., 1968; Leist et al., 1995), the liver tissue was observed to evaluate the effect of pirfenidone treatment (pre or post lipopolysaccharide/D-galactosamine challenge). In the vehicle-treated group, the liver sections were observed to have broad hemorrhagic necrosis 6 h after lipopolysaccharide/D-galactosamine challenge (Fig. 6B), in which apoptotic positive hepatocytes were also observed with Tunel staining (the nucleus was stained brown, as shown in Fig. 6F). In mice receiving pirfenidone pre or post lipopolysaccharide/D-galactosamine challenge, surprisingly, the histopathological sections were almost the same as those of normal mice with HE staining (Fig. 6C,D). Apoptotic hepatocytes were also

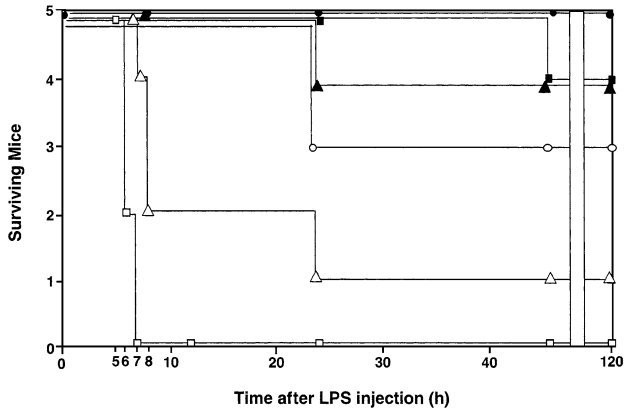


Fig. 5. Posttreatment effect of pirfenidone on lethality of lipopolysaccharide/D-galactosamine-challenged mice. All mice were injected i.p. with lipopolysaccharide (50 μg/kg) and D-galactosamine (250 mg/kg). A single pirfenidone (500 mg/kg, p.o.) was administered at 1, 2, 3, 4 or 5 h after lipopolysaccharide/D-galactosamine challenge ($n=5$/group). Pirfenidone was suspended in 200 μl of 0.5% CMC vehicle. All vehicle-treated mice died during the first 12 h after lipopolysaccharide/D-galactosamine challenge. Surviving mice were monitored for 120 h. Open squares, vehicle treated; closed circles, pirfenidone treated 1 h post-lipopolysaccharide/D-galactosamine challenge; closed squares, pirfenidone treated 2 h post-lipopolysaccharide/D-galactosamine challenge; closed triangle, pirfenidone treated 3 h post-lipopolysaccharide/D-galactosamine challenge; open circle, pirfenidone treated 4 h post-lipopolysaccharide/D-galactosamine challenge; open triangle, pirfenidone treated 5 h post-lipopolysaccharide/D-galactosamine challenge.

negative in these pirfenidone-treated tissues with Tunel staining (Fig. 6G,H). From these results, pirfenidone treatment before or after lipopolysaccharide/D-galactosamine challenge appeared to be effective for suppressing apoptotic cell death and liver failure.

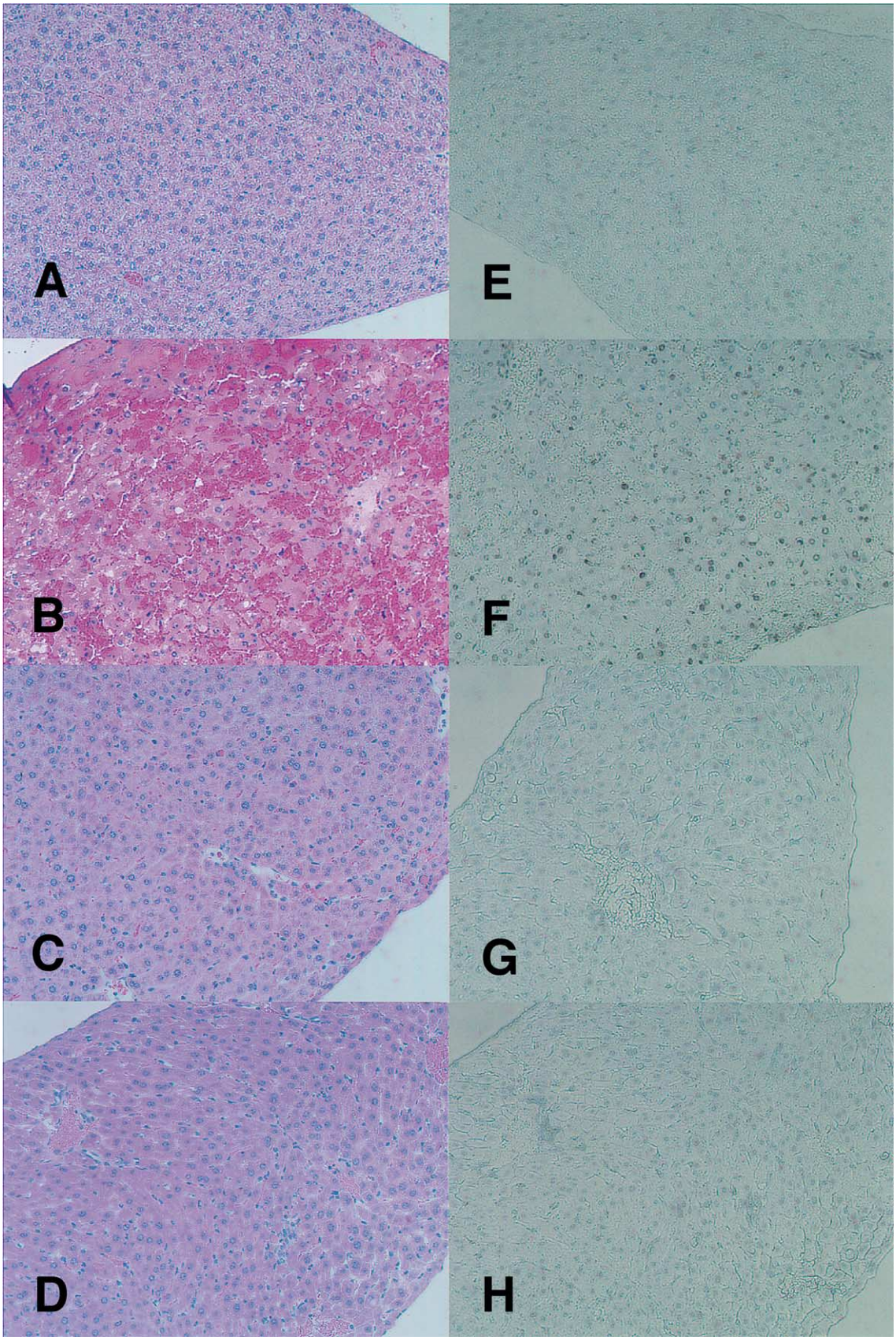
3.7. TGF-β1 levels in the liver tissues

To investigate the relationship between anti-inflammatory properties and anti-fibrotic properties of this agent,

Table 1
Effect of interferon-γ injection on lethality of pre/post pirfenidone-administered mice

Group	Pretreatment (pirfenidone)	Posttreatment (pirfenidone)	Postinjection (interferon-γ)	Lethality (%) (n/10(5))
I	−	−	−	100 (5/5)
II	+	−	−	0 (0/5)
III	−	+	−	10 (1/10)
IV	+	−	+	0 (0/10)
V	−	+	+	50 (5/10)

All mice were injected i.p. with lipopolysaccharide (50 μg/kg) and D-galactosamine (250 mg/kg). Pirfenidone (500 mg/kg, p.o.) was administered pre or post lipopolysaccharide/D-galactosamine challenge. Pretreatment was the administration of pirfenidone 5 min before lipopolysaccharide/D-galactosamine challenge. Posttreatment was the administration of pirfenidone 3 h after lipopolysaccharide/D-galactosamine challenge. Interferon-γ injection (i.p.) was done 4 h after lipopolysaccharide/D-galactosamine challenge. The survival of mice was observed after 5 days of lipopolysaccharide/D-galactosamine challenge.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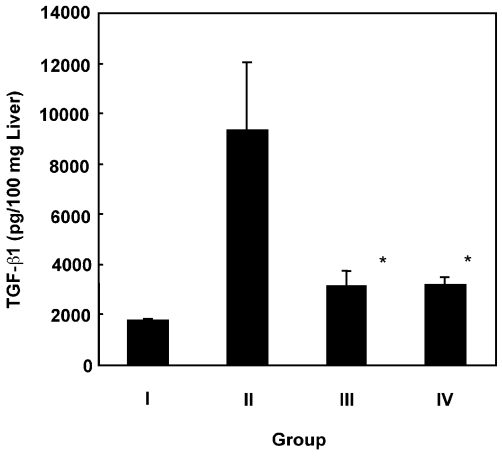

Fig. 7. Prophylactic or therapeutic effect of pirfenidone against lipopolysaccharide/D-galactosamine-induced TGF-β1 elevation in the liver tissues. Four groups of mice lipopolysaccharide/D-galactosamine-challenged or not (control) were treated with pirfenidone (500 mg/kg, p.o., $n=3$/group). Normal control mouse (group I), 0.5% CMC vehicle administration 5 min before lipopolysaccharide/D-galactosamine challenge (group II), pirfenidone administration 5 min before lipopolysaccharide/D-galactosamine challenge (group III) and pirfenidone administration 4 h after lipopolysaccharide/D-galactosamine challenge (group IV), respectively. At 6 h after lipopolysaccharide/D-galactosamine challenge, liver specimens were obtained and kept in a deep freezer (-80 °C). The frozen liver specimen (approximately 100 mg) was homogenized and sampled to TGF-β1 ELISA kit as described in Materials and methods. Each column represents the mean ± S.D. $*P<0.01$ (Student's t-test) significantly different as compared to vehicle-treated group (group II).

TGF-β1 levels in the liver tissues were determined. The liver tissue was homogenized and lysed (100 mg tissue/ml of lysis buffer), and the lysate was sampled to TGF-β1 ELISA kit. The results are shown in Fig. 7. In saline-/vehicle-treated group I, TGF-β1 level was 1799 ± 65 pg/100 mg liver tissue; however, it was markedly elevated to 9341 ± 2662 pg/100 mg liver tissue in lipopolysaccharide/D-galactosamine-injected group II. This elevation was extremely suppressed in pre or post pirfenidone-treated groups (III and IV). In the liver of mice receiving pirfenidone pre lipopolysaccharide/D-galactosamine challenge, the TGF-β1 level was 3122 ± 622 pg/100 mg liver tissue (83% suppression). In the liver of mice receiving pirfenidone post lipopolysaccharide/D-galactosamine challenge, the TGF-β1 level was 3194 ± 297 pg/100 mg liver tissue (82% suppression). From these results, lipopolysaccharide/D-galactosamine challenge induced marked hemorrhagic necrosis and TGF-β1 elevation in the liver, and pirfenidone treatment before or after lipopolysaccharide/D-galactosamine challenge appeared to be effective for suppressing these remarkable TGF-β1 elevation in the liver tissues.

4. Discussion

Pirfenidone (S-7701) is known to be a broad spectrum anti-fibrotic agent, a non-peptide and small molecular weight molecule (m.w. of 185.22) as shown in Fig. 1. This compound is underdevelopment for clinical use against idiopathic pulmonary fibrosis (Raghu et al., 1999), scleroderma, multiple sclerosis and sclerosing peritonitis (Suga et al., 1995; Taniyama et al., 1997). Pirfenidone can safely arrest further progression of existing fibrotic lesions (Iyer et al., 1995; Margolin and Lefkowitz, 1994), reduce or remove excessive fibrotic lesions or scar tissue and prevent the development of fibrotic lesions (Margolin and Lefkowitz, 1994). In relation to the mechanism, pirfenidone has been shown to modify significant cytokine regulatory actions (especially that of platelet-derived growth factor (PDGF), TGF-β1, fibroblast growth factor (bFGF), epidermal growth factor (EGF) and TNF-α) and to inhibit fibroblast proliferation and collagen matrix synthesis. However, the detailed mechanism remains unknown. We compared the efficacious doses of pirfenidone administration in each animal disease model. In chronic fibrotic disease models, the dose of administration was reported to be 500–750 mg/kg/day p.o. in cyclophosphamide-induced murine lung fibrosis (Kehrer et al., 1997), 500 mg/kg/day p.o. in asbestos-induced hamster lung fibrosis (Margolin et al., 1982), 500 mg/kg/day p.o. in bleomycin-induced lung fibrosis (Iyer et al., 1995), 500 mg/kg/day p.o. in rat experimental renal fibrosis (Shimizu et al., 1997, 1998) and 350 mg/kg/day p.o. in chlorhexidine gluconate-induced experimental sclerosing peritonitis model (Suga et al., 1995). For an acute inflammatory disease model, the dose of administration was more than 200 mg/kg i.p. for the murine endotoxic shock model (Cain et al., 1998). In both chronic and acute disease models, the efficacious doses showed good correspondence—more than 200 mg/kg of pirfenidone administration by p.o. or i.p. route. From these findings, we hypothesized that the anti-fibrotic and anti-inflammatory actions are caused by the same mechanism of this agent. Thus, the anti-inflammatory actions of this agent were studied to evaluate the functional mechanism using the murine acute endotoxic shock model.

A prophylactic effect and a therapeutic effect of pirfenidone on murine endotoxic lethal shock are proposed in this

Fig. 6. Prophylactic or therapeutic effect of pirfenidone against lipopolysaccharide/D-galactosamine-induced liver injury by microscopic observation. Groups of four mice lipopolysaccharide/D-galactosamine-challenged or not (control) were treated with pirfenidone (500 mg/kg, p.o.). (A) Normal control mouse, (B) 0.5% CMC vehicle administration 5 min before lipopolysaccharide/D-galactosamine challenge, (C) pirfenidone administration 5 min before lipopolysaccharide/D-galactosamine challenge and (D) pirfenidone administration 4 h after lipopolysaccharide/D-galactosamine challenge, respectively. At 6 h after lipopolysaccharide/D-galactosamine challenge, liver specimens were sampled for histological studies. A through D represent typical results of HE staining for the liver specimens. E through H represent the results of Tunel staining for A through D, respectively. Original magnification × 100.

report. The prophylactic effect can be explained from the aspect of a regulatory action on various inflammatory cytokines. From reported findings, high levels of circulating TNF-α, interleukin-1, interleukin-6, interleukin-10, interleukin-12 and interferon-γ exist during endotoxemia (van Deuren et al., 1992). Among these cytokines, sequential TNF-α, interleukin-12, interferon-γ and interleukin-10 are key factors in the development of septic disease (Heinzel et al., 1994; Ozmen et al., 1994; Wysocka et al., 1995). The endotoxin-induced initial event is the production of pro-inflammatory cytokine TNF-α, which causes production of interleukin-12 (and interleukin-18), and interferon-γ is induced. Interferon-γ furthermore induces the Fas–Fas ligand system in liver tissue and acute hepatocyte apoptosis is induced, causing hemorrhagic necrotic failure and lethal shock (Tsutsui et al., 1996, 1997). This sequential cascade was reported to include more critical events than other inflammatory cytokines, interleukin-1, interleukin-6, interleukin-8 and GM-CSF (Ozmen et al., 1994; Wysocka et al., 1995). In our study, pirfenidone administration decreased TNF-α, interleukin-12 and interferon-γ in lipopolysaccharide-injected mice, and also decreased interleukin-18 in lipopolysaccharide shock mice primed with heat-killed *Propionibacterium acnes* (data not shown). In contrast, pirfenidone did not decrease interleukin-1 and interleukin-6 as much as those of TNF-α, interleukin-12 and interferon-γ. Interleukin-10 is known as an anti-inflammatory cytokine that suppresses the serum level of TNF-α and lethal shock in endotoxin-challenged mouse (Fiorentino et al., 1991; Howard et al., 1993). In our study, pirfenidone markedly enhanced the serum interleukin-10 level. Therefore, the prophylactic effect is considered to originate from suppression of the sequential cytokine cascade of TNF-α, interleukin-12 (interleukin-18) and interferon-γ, and enhancement of the interleukin-10 level. This cytokine regulatory profile is very attractive for clinical use. Recently, evidence has been presented that anti-TNF-α and soluble TNF-α receptors are effective against rheumatoid arthritis (Newton and Decicco, 1999). Recombinant human interleukin-10 has been clinically effective against steroid-resistant Crohn's disease (VanDaventer et al., 1997). The cytokine regulatory action of pirfenidone, down-regulation of TNF-α and up-regulation of interleukin-10 may be beneficial for therapy of TNF-α-associated diseases such as rheumatoid arthritis, Crohn's disease and septic shock syndrome.

The therapeutic effect of pirfenidone post lipopolysaccharide challenge was revealed by this study. Pirfenidone prevented the death of mice even when given 3 or 4 h after lipopolysaccharide challenge. This effect cannot be explained by an inhibitory effect on TNF-α production because TNF-α had already increased and then disappeared by this time. Thus, this effect was considered not to be mediated through TNF-α. We observed serum levels of other cytokines, interleukin-12, interleukin-10 and interferon-γ in this therapeutic process. Only interferon-γ was suppressed. Suppression of interferon-γ production is possibly involved in the posttreatment effect by pirfenidone. On the histopathological level, the apoptotic positive cells were extremely reduced and acute hepatitis was evidently suppressed in the pirfenidone posttreated murine liver, as well as in the pretreated one. Pirfenidone may play a role not only in regulating inflammatory cytokines but also suppressing subsequent apoptotic processes in the liver. These results may offer an alternative for the treatment of human septic shock syndrome and acute hepatitis by pirfenidone.

The pathogenesis of fibrosis is poorly understood. In general, the series of events can be divided into three stages: (1) the inflammatory process, (2) tissue injury and (3) excessive tissue remodeling process, that is, fibrosis. In the fibrotic process, the involvement of TGF-β, FGF and vascular endothelial growth factor (VEGF), the growth of fibroblasts and the increase of collagen deposition are suspected (Colby and Churg, 1986; Bowden, 1984; Reiser and Last, 1986; Kovacs and Kelly, 1985; Suwabe et al., 1988). Also, the inflammation and tissue injury are important for the induction of tissue remodeling in the late stage and fibrotic process. One of the critical factors in the inflammatory process is TNF-α. The lungs of mice receiving bleomycin has been reported to express TNF-α and pulmonary fibrosis in these mice could be reduced by injection of anti-TNF-α antibody (Piguet et al., 1989). Another report showed that lung fibrosis was evident in TNF-α transgenic mice (Sueoka et al., 1998). TNF-α has a wide range of activities (Beutler and Cerami, 1989), one of these was being a growth-promoting activity on fibroblasts which leads to increased local collagen deposition. Therefore, TNF-α is also associated with the pathogenesis of various fibrotic diseases. Recently, the association of interleukin-10 with fibrosis was also reported. Arai et al. (2000) reported that introduction of the interleukin-10 gene into mice-inhibited, bleomycin-induced lung injury in vivo. Nelson et al. (2000) reported that interleukin-10 treatment reduced fibrosis in patients with chronic hepatitis C. These observations suggest that TNF-α-suppressing and interleukin-10-enhancing activities may contribute to the anti-fibrotic actions of pirfenidone. TGF-β is known as a most important fibrogenic cytokine in the fibrotic process. We observed remarkable elevation of TGF-β1 level in injured liver tissue induced by lipopolysaccharide/D-galactosamine and suppression in that of mice pre- or posttreated by pirfenidone (Fig. 7). The mechanism of the TGF-β1 suppression was not cleared yet, but we considered that pirfenidone protects murine liver tissue from hemorrhagic and necrotic injury by the anti-inflammatory effects, and the marked elevation of TGF-β1 level is suppressed. The anti-fibrotic properties of pirfenidone may be associated with the anti-inflammatory properties through the anti-inflammatory or tissue protective activity, and the suppression of tissue TGF-β1 elevation. Warshamana et al. (2001) reported that TNF-α induces TGF-β1. Suppression of TNF-α production by pirfenidone may cause the suppression of tissue TGF-β1 elevation.

In conclusion, the novel anti-fibrotic agent pirfenidone showed several unique anti-inflammatory properties. It suppressed inflammatory cytokines, TNF-α, interleukin-12, interferon-γ and enhanced interleukin-10 levels in lipopolysaccharide/D-galactosamine-injected mice and also offered protection against subsequent lethal shock. Furthermore, pirfenidone showed a therapeutic effect post lipopolysaccharide challenge and suppressed apoptosis, hemorrhagic necrotic liver failure and marked elevation of tissue TGF-β1. These findings suggest that pirfenidone should be beneficial against a wide variety of acute or chronic inflammatory diseases as well as fibrotic diseases.

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