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Modulation of airway remodeling-associated mediators by the antifibrotic compound, pirfenidone, and the matrix metalloproteinase inhibitor, batimastat, during acute lung injury in mice

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Abstract

Matrix metalloproteinases (MMPs) are potent to degrade basement membrane collagen associated with acute lung injury in inflammatory processes. We have investigated effects of pirfenidone, antifibrotic agent, and batimastat, inhibitor of MMPs, on gelatinase activities, on release of tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β), as well as on recruitment of inflammatory cells in bronchoalveolar lavage (BAL) fluid after aerosol administration of lipopolysaccharide (LPS) in mice. Pretreatment with pirfenidone reduced neutrophil recruitment, TNF-α and TGF-β levels, and MMP-9 secretion. In contrast, pretreatment with batimastat (30 or 60 mg/kg, i.p.) only reduced TNF-α and TGF-β levels. Batimastat did not reduce MMP secretion in BAL fluid but inhibited MMP-9 activity. The increase in tissue inhibitor of matrix metalloproteinase (TIMP)-1 induced by LPS was not modified by the two drugs. These findings demonstrate that the two drugs can inhibit the in vivo increase in MMP induced by LPS, batimastat with a direct inhibitor effect on MMP activity and pirfenidone as a consequence of its antiinflammatory effect.

Keywords: Lipopolysaccharide; Pirfenidone; Batimastat; Bronchoalveolar lavage; Matrix metalloproteinase (MMP); TIMP (tissue inhibitor of matrix metalloproteinase)

1. Introduction

Acute lung injury is characterized by high microvascular permeability, low pressure pulmonary edema, refractory hypoxemia, and respiratory failure. The onset of acute lung injury is often an early symptom of multiple organ failure associated with sepsis, and sepsis is associated with elevated blood levels of endotoxin or lipopolysaccharide (LPS). Indeed, the lung is known to be a prime target organ for LPS derived from Gram-negative bacteria (Parsons et al., 1989). Gram-negative sepsis in humans often leads to lung complications and, in the worst case, acute respiratory distress syndrome (ARDS). Therefore, the LPS-induced acute inflammation model is well adapted to the study of the pathogenesis of ARDS (Van Helden et al., 1997). The sequestration of neutrophils in the pulmonary microcirculation and their activation appears to be a key event in the development of lung injury (Worthen et al., 1987). The sequestered neutrophils, when activated, are a source of several inflammatory mediators that can contribute to the destruction of the basement membrane as observed in ARDS (Kollef and Schuster, 1995) and that can lead to pulmonary fibrosis (Crouch, 1990).

Basement membranes are the thin extracellular matrices underlying most epithelium and endothelium that play a major role in various biological processes, particularly tissue remodeling after parenchymal damage. Extracellular matrix degradation is controlled primarily by matrix metalloproteinases (MMPs), a family of zinc-dependent secreted enzymes that, collectively, are capable of degrading the major components of the matrix such as collagens, gelatins

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and proteoglycans. After secretion in a latent form, MMP activities are regulated by proteolytic activation of the proenzymes and by interaction of the active form with their specific inhibitors (tissue inhibitors of matrix metalloproteinases (TIMP)) (Birkedal-Hansen, 1995). With regard to the lung, MMPs have been implicated in the pathophysiology of lung cancer, and acute and chronic inflammatory diseases including ARDS (Ricou et al., 1996; Lanchou et al., 2000) and interstitial lung fibrosis (Pardo et al.,1992; Fukuda et al., 1998). Of the MMP family, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) cleave type IV collagen, which is an important constituent of basement membrane. Recently, we have reported the development of pulmonary fibrosis induced by chronic exposure to LPS in mice. In this model, collagen deposition in lung was associated with an increase in gelatinase (MMP-2 and MMP-9) activity in bronchoalveolar lavage (BAL) (Corbel et al., 2001a).

Using a murine model of acute lung injury or asthma, we previously demonstrated that anti-inflammatory drugs, namely corticosteroids and selective phosphodiesterase type 4 inhibitor were able to inhibit MMP-9 and MMP-2 activities (Corbel et al., 1999; Germain et al., 2000; Belleguic et al., 2000), suggesting that these compounds may modulate early airway remodeling associated with the inflammatory process. Since fibrosis is generally a final outcome of the airway inflammatory process, the aim of this study was to investigate the effects of two compounds, the anti-fibrotic compound, pirfenidone, and the MMP inhibitor, batimastat, on inflammation and airway remodeling-associated mediators namely, tumor necrosis factor-α (TNF-α), gelatinase (MMP-9 and MMP-2) activity and transforming growth factor-β (TGF-β) following acute lung injury induced by LPS exposure aerosol in mice. Indeed, Pirfenidone has been reported to be effective to both prevent and treat bleomycin-induced lung fibrosis in hamsters (Iyer et al., 1995, 1998). We recently reported that batimastat (30 mg/kg), a hydroxamate-based MMP inhibitor, was efficient to reduce pulmonary fibrosis induced by bleomycin in mice (Corbel et al., 2001b).

2. Materials and methods

2.1. Materials

Lipopolysaccharide from *Escherichia coli* (0.55 B5), gelatin and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). May–Grünwald and Giemsa stains were from RAL (Paris, France). Sodium pentobarbital was from Sanofi santé animale (Libourne, France). Acrylamide was from ICN (Aurora, OH, USA). Coomassie blue was from Biorad (München, Germany). Sodium dodecyl sulfate (SDS) and Tris solution were from Eurobio (Les Ulis, France). Mouse monoclonal TGF-β antibody was provided by R&D System (Minneapolis, MN, USA). Mouse monoclonal TIMP-1 antibody was purchased from Novo Castra (Newcastle, UK). Pirfenidone was purchased from Tocris (Ballwin, MO, USA) and batimastat (BB-94) was a generous gift from Dr. Helen R. Mills (British Biotechnology, Oxford, UK).

2.2. Treatment of mice and LPS exposure

Ten-week-old male Balb/c mice were purchased from CERJ (Le Genest Saint Isle, France). The mice were allowed to acclimate to the facilities for 1 week prior to any treatment. They were raised and housed in our standard animal care facilities. A 12 h/12 h light/dark cycle was maintained. All mice were fed standard pellet food (UAR, Villemoisson-sur-Orge, France) and given water ad libitum. The mice were exposed for 60 min to an aerosol of LPS (100 μg/ml) in saline solution (0.9% NaCl) or to an aerosol of the saline solution alone. For exposure, non-anaesthetized mice were placed in a Plexiglass chamber ($30 \times 50 \times 30$ cm) directly connected to a De Vilbiss ultrasonic nebulizer (Ultraneb 99, Sommerset, PA, USA) that generated particles with an aerodynamic diameter averaging 0.5–3 μm.

Batimastat was sonicated into suspension with phosphate buffer saline (PBS) and 0.01% Tween 80. The animals were given by an intraperitoneal (i.p.) injection of 30 or 60 mg/kg batimastat or an equal volume of vehicle (PBS, 0.01% Tween 20) 24 h and 30 min before LPS exposure. In another set of experiments, mice were given an i.p. injection of 20, 100 or 200 mg/kg pirfenidone or an equal volume of vehicle (NaCl 0.9%, 10% ethanol) 24 and 2 h before LPS exposure. BAL were collected 24 h after LPS exposure.

2.3. Bronchoalveolar lavage

After semi-excision of the trachea, a plastic cannula was inserted and the airspace was washed with 0.5 ml of 0.9% NaCl with a 1-ml syringe. This operation was repeated 10 times in same mouse. BAL was centrifuged ($600 \times g$ for 10 min, 4 °C) and the fluid phase of the first ml of BAL fluid was divided into aliquots and frozen at −80 °C. After lysis of erythrocytes with distilled water, cell pellets were resuspended in 500 μl 0.9% NaCl.

2.4. Bronchoalveolar lavage cell evaluation

A total cell count was obtained with a hemacytometer chamber, and viability was determined by trypan blue exclusion. BAL cells were adjusted to a concentration of 5×10^5 cells/ml in 0.9% NaCl. After cytocentrifugation (Cytopro 7620 WESCOR) at 700 rpm for 10 min, the cells were stained with May–Grünwald Giemsa. Differential counts were made with 200 cells using standard morphological criteria.

2.5. TNF-α and TGF-β measurements

The amount of tumor necrosis factor-α (TNF-α) in BAL fluid was quantified by enzyme-linked immunosorbent assay (ELISA) (Genzyme, Cambridge, USA).

For TGF-β evaluation, standardized protein quantities of BAL fluids were loaded to SDS-PAGE under reducing conditions, and were transferred electrophoretically onto nitrocellulose membranes (Hybond-ECL, Amersham Life Science, UK). The filters were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS), then were incubated with anti-mouse TGF-β monoclonal antibody diluted in TBS containing 0.1% bovine serum albumine (BSA) and 0.3% Tween 20 (T). The filters were then washed three times for 10 min in TBS and incubated with peroxidase-conjugate immunoglobulin G (IgG). All incubations were performed at room temperature for 2 h. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, UK) and the blots were exposed to X-ray films. The results were expressed as percentages of the intensity of a sample loaded onto each filter. This sample, BAL from one mouse exposed to LPS, was loaded onto each filter and used as an internal standard of intensity in order to allow comparison between filters. Under reducing conditions, this antibody detects the active TGF-β homodimer (25 kDa).

2.6. Gelatin zymography

Aliquots of BAL fluid were subjected to electrophoresis on a 4.5% acrylamide stacking gel/7% acrylamide-separating gel containing 1 mg/ml gelatin, in the presence of sodium dodecyl sulfate (SDS), under non-reducing conditions as previously described (Corbel et al., 1999). After electrophoresis, gels were washed twice with 2.5% Triton X-100, rinsed with water and incubated at 37 °C overnight in 50 mM Tris, 5 mM $CaCl_2$, 1 nM $ZnCl_2$, pH 8 (incubation buffer). The gels were stained with Coomassie Brilliant Blue and destained in a solution of 25% ethanol and 10% acetic acid. Gelatinase activities appeared as clear bands against a blue background. Enzyme amounts were quantified by measuring the intensity of the negative bands using densitometric analysis with "Densylab" software (Bioprobe Systems, Les Ulis, France). The results were expressed as percentages of the intensity of a sample loaded onto each zymogram. This sample, a BAL from one mouse exposed to LPS, was loaded onto each gel and used as an internal standard of intensity in order to allow comparison between zymograms.

For some samples of BAL fluid, batimastat (500 nM) was added to the incubation buffer to study the MMP inhibitory effect of batimastat in comparison with that of pirfenidone (1.6 mM).

2.7. Western blot analysis for TIMP-1

Standardized protein quantities of BAL fluids were loaded on to SDS-PAGE under reducing conditions, and were transferred electrophoretically onto nitrocellulose membranes (Hybond-ECL, Amersham Life Science). After the filters were blocked with 5% non-fat milk in Tris-buffered saline/0.1% Tween 20 (TBS-T), they were incubated 2 h at room temperature with diluted TIMP-1 monoclonal antibody in TBS-T containing 1% BSA The filters were then washed three times for 15 min in TBS-T and incubated 2 h at room temperature with corresponding peroxidase-conjugate IgG. Membranes were washed four times in TBS-T for 15 min, and specific bands were visualized using the ECL system from Amersham according to the manufacturer's instructions.

Table 1
Influence of batimastat (BAT) or pirfenidone (PFD) on LPS-induced changes in cell composition in BAL fluid of Balb/c mice (cells $\times 10^5 \pm$ SEM)

	Treatment	*n*	Total cells	Macrophages	Neutrophils	Lymphocytes
sal	veh	7	4.09 ± 0.77	4.05 ± 0.77	0.02 ± 0.01	0.01 ± 0.005
sal	PFD 200	4	4.07 ± 0.33	4.06 ± 0.33	0	0.004 ± 0.004
LPS	veh	10	19.96 ± 1.76[a]	3.23 ± 0.48	16.58 ± 1.69[a]	0.13 ± 0.062[a]
LPS	PFD 20	5	24.52 ± 3.91	3.7 ± 0.69	17.98 ± 4.9	0.09 ± 0.07
LPS	PFD 100	5	17.68 ± 3.1	4.4 ± 0.7	10.73 ± 2.78	0.19 ± 0.08
LPS	PFD 200	8	9.04 ± 1.41[b]	3.26 ± 0.35	5.72 ± 1.25[c]	0.07 ± 0.02
sal	veh	6	3.84 ± 0.54	3.56 ± 0.76	0.006 ± 0.004	0.001 ± 0.001
sal	BAT 60	3	3.8 ± 0.51	3.8 ± 0.051	0	0
LPS	veh	7	29.05 ± 3.91[a]	2.74 ± 0.59	22.37 ± 5.54[a]	0.03 ± 0.03
LPS	BAT 30	6	21.86 ± 4.27	2.68 ± 0.69	17.20 ± 5.5	0.05 ± 0.05
LPS	BAT 60	6	24.43 ± 2.52	3.06 ± 0.68	20.96 ± 2.46	0.08 ± 0.04

Pirfenidone (PFD) was administrated i.p. at the dose of 20, 100 or 200 mg/kg, 24 and 2 h before the LPS aerosol (100 μg/ml for 60 min). Batimastat (BAT) was administrated i.p. at the dose of 30 or 60 mg/kg, 24 and 1 h before the LPS aerosol. *n* = number of mice.

[a] $P < 0.01$ in comparison with control non-treated mice exposed to a saline solution alone (sal-veh).

[b] $P < 0.01$ in comparison with control non-treated mice exposed to LPS aerosol (LPS-veh).

[c] $P < 0.05$ in comparison with control non-treated mice exposed to LPS aerosol (LPS-veh).

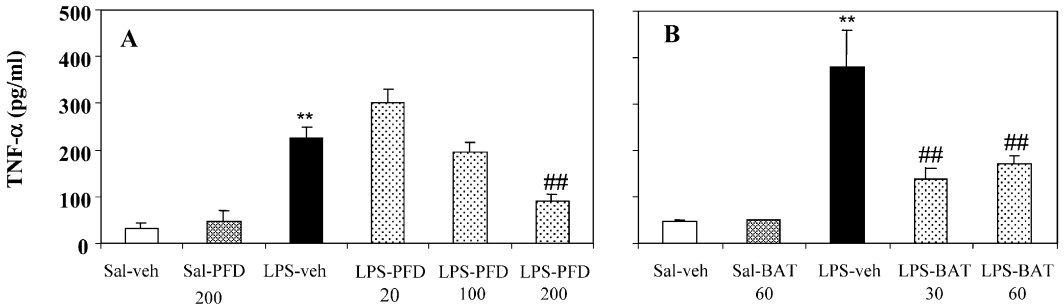

Fig. 1. Levels of TNF-α in BAL fluid from mice treated with either pirfenidone (PFD; 20, 100 or 200 mg/kg) (LPS-PFD), or batimastat (BAT; 30 or 60 mg/kg) (LPS-BAT) before LPS aerosol or saline exposure (sal-PFD or sal-BAT). BAL was obtained 24 h after LPS exposure. The results are presented as means ± SEM. $^{**}P < 0.01$ in comparison with control non-treated mice exposed to a saline solution alone (sal-veh); $^{\#\#}P < 0.01$ in comparison with control non-treated mice exposed to LPS aerosol (LPS-veh). Number of mice in each group is indicated in Table 1.

2.8. Expression of the results and statistical analysis

The results are expressed as means ± standard error of mean (SEM). Statistical analysis was performed with the "Statview" software on an Apple computer. Analysis of treatment effects between the various groups was performed with a two-way ANOVA. Comparison of treatment interaction was done with Newman–Keuls tests. For each analysis, P values less than 0.05 were considered statistically significant.

3. Results

3.1. Total number of cells and cellular composition in BAL

Exposure to LPS aerosol led to a significant increase in the total number of BAL cells, compared with that of saline-exposed mice (sal-veh) (Table 1). The most significant increase in number of cells after LPS exposure in mice was noted for neutrophils whereas the number of macrophages in the BAL was not modified whatever the group of mice. Lymphocytes were only significantly increased after LPS exposure in the group of experiments concerning pirfenidone effect study. Treatment of mice with pirfenidone (200 mg/kg) produced a significant inhibition of the increase in the total number of BAL cells and neutrophils following LPS exposure alone. In contrast, batimastat did not significantly affect the increase in total number of cells and neutrophils.

3.2. Cytokine measurements

The level of TNF-α in BAL fluid was significantly increased after LPS challenge in comparison to that in saline-exposed mice (sal-veh). The LPS-induced increase in TNF-α level was significantly reduced in BAL fluid from mice treated with either pirfenidone (200 mg/kg) or batimastat (30 and 60 mg/kg) whereas the basal TNF-α level was not modified after treatment with either of the drugs (Fig. 1).

BAL fluid from mice exposed to saline and treated with vehicle (sal-veh) or pirfenidone (200 mg/kg) (sal-PFD 200) or batimastat (60 mg/kg) (sal-BAT 60) mice contained similar low levels of TGF-β, as measured by densitometric analysis of Western blot (Fig. 2). In contrast,

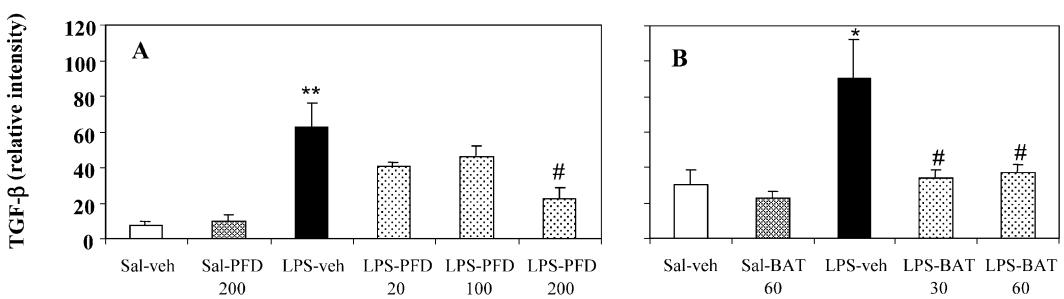

Fig. 2. Levels of TGF-β in BAL fluids from mice treated with either pirfenidone (PFD; 20, 100 or 200 mg/kg) (LPS-PFD) or batimastat (BAT; 30 or 60 mg/kg) (LPS-BAT) before the LPS aerosol or saline exposure (sal-PFD or sal-BAT). BAL was obtained 24 h after LPS exposure. The results are presented as means ± SEM of relative percentage of an internal standard loaded onto each gel. $^{**}P < 0.01$; $^{*}P < 0.05$ in comparison with control non-treated mice exposed to a saline solution alone (sal-veh); $^{\#}P < 0.05$ in comparison with control non-treated mice exposed to LPS aerosol (LPS-veh). Number of mice in each group is indicated in Table 1.

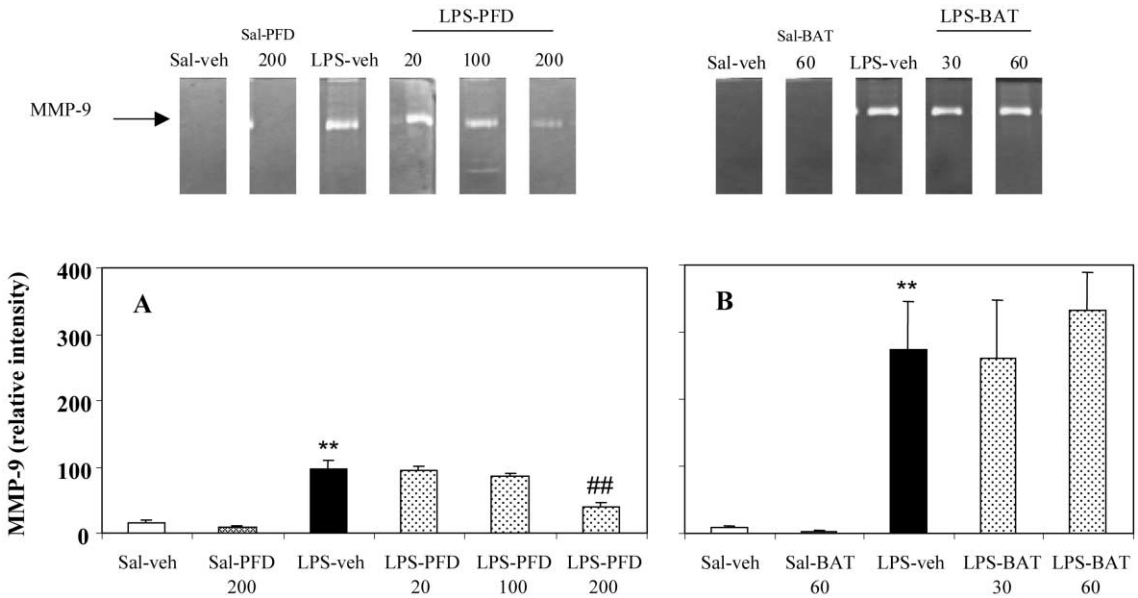

Fig. 3. Upper panel: Gelatin zymogram performed with samples of BAL fluids subjected to electrophoresis. Gels were then washed twice with 2.5% triton X-100 and incubated at 37 °C overnight in incubation buffer. After staining with coomassie brillant blue, gels were destained in a solution of 25% ethanol and 10% acetic acid. Proteolysis areas appeared as clear bands against a blue background. Lower panel: Effects of pirfenidone (PFD) or batimastat (BAT) on MMP-9 activity in BAL fluid from mice before exposure to LPS aerosol. MMP-9 activity was determined using gelatin zymography and the intensity of these activities was measured by densitometry. BAL was obtained 24 h after LPS exposure. The results are presented as means ± SEM of relative percentages of a band of migration of an internal standard loaded onto each gel. $^{**}P < 0.01$ in comparison with control non-treated mice exposed to a saline solution alone (sal-veh); $^{\#\#}P < 0.01$ in comparison with control non-treated mice exposed to LPS aerosol (LPS-veh). Number of mice in each group is indicated in Table 1.

LPS aerosol administration led to an increase in TGF-β in the BAL fluid. Moreover, treatment of mice with pirfenidone (200 mg/kg) or batimastat (30 and 60 mg/kg) significantly inhibited the increase in TGF-β level in BAL fluid after LPS exposure.

3.3. Metalloproteinase activity in BAL fluids

The effects of pirfenidone and batimastat on gelatinase activity in BAL fluid from mice exposed to LPS are summarized in Fig. 3, illustrated by both zymogram (upper panel) and densitometric analysis of zymogram (lower panel). MMP-9 gelatinolytic activity (92 kDa) was significantly increased in LPS-treated mice (LPS-veh) in comparison to that in control mice treated or not with pirfenidone or batimastat (sal-veh; sal-PFD 200; sal-BAT 60, respectively). Only the highest dose of pirfenidone (200 mg/kg) elicited a significant reduction of the increase in MMP-9 activity in BAL fluids from mice exposed to LPS aerosol (LPS-PFD 200) (Fig. 3).

3.4. Inhibition of gelatinases in vitro

In this set of experiments, we wished to demonstrate that batimastat directly inhibits gelatinase activity. BAL fluids from mice were run on a zymography gel, and gelatinolytic activity was analyzed in vitro by incubating the gels overnight in incubation buffer containing batimastat or pirfenidone, or in incubation buffer alone. When batimastat was added to the incubation buffer no lytic bands were seen, confirming the direct inhibition of MMP activity by batimastat (Fig. 4).

3.5. TIMP activity in BAL fluids

A significant increase in TIMP-1 signal was observed in BAL fluid collected from LPS-exposed mice (LPS-veh) (Fig. 5) in comparison to fluid from control mice (sal-veh or sal-PFD 200 and sal-BAT 60). No decrease in TIMP-1

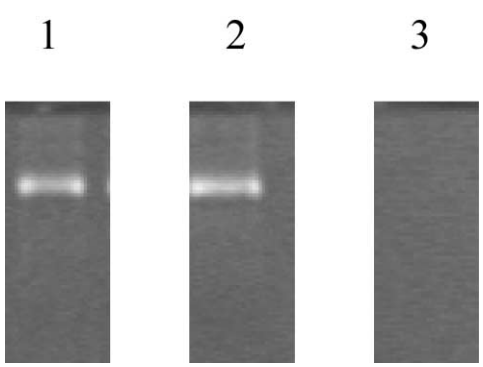

Fig. 4. Gelatin zymogram with samples of BAL fluids from mice exposed to LPS (LPS-veh). After migration, pirfenidone (PFD, 1.6 mM) or batimastat (BAT, 500 nM) was added to the incubation buffer. Lane 1: incubation buffer alone; lane 2: incubation buffer with PFD; lane 3: incubation buffer with BAT.

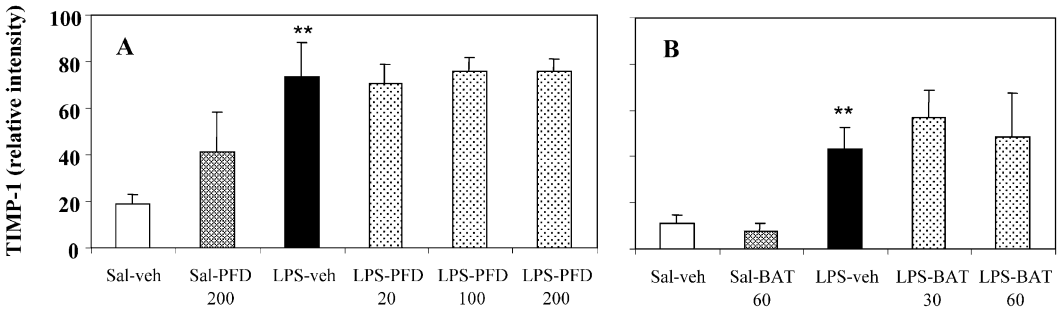

Fig. 5. TIMP-1 level in BAL fluids. TIMP-1 was quantified by immunoblotting and the intensity of signals was measured by densitometric analysis. Results were expressed as percentages of internal standard loaded onto each gel. The results are presented as means ± SEM. $^{**}P < 0.01$ in comparison with control non-treated mice exposed to a saline solution alone (sal-veh). Number of mice in each group is indicated in Table 1.

was observed after treatment of mice with pirfenidone or batimastat.

4. Discussion

In the present study, we were interested in the activity of two recently developed drugs, pirfenidone and batimastat, which were reported to be effective to reduce the development of pulmonary fibrosis. Pirfenidone, an investigational antifibrotic drug, was reported to offer protection against the development of lung fibrosis in the bleomycin-treated hamster model (Iyer et al., 1995, 1998). Pirfenidone is also currently undergoing clinical trials in patients with advanced idiopathic pulmonary fibrosis (Raghu et al., 1999). We recently demonstrated that the synthetic MMP inhibitor, batimastat, which has been developed for cancer research (Denis and Verweij, 1997), at the dose of 30 mg/kg could inhibit the development of bleomycin-induced pulmonary fibrosis in mice (Corbel et al., 2001b). Since pulmonary fibrosis is a consequence of many types of severe lung injury and is almost always associated with an alveolar inflammation reaction, these results prompted us to examine the effect of these drugs on the release of airway remodeling-associated mediators during acute lung injury.

We have previously described a murine model of acute lung injury following exposure to a single aerosol of LPS (Corbel et al., 1999). In this model, we characterized the relationship between the inflammatory process and MMP activity in BAL fluid from mice and this model was used to investigate the effects of pirfenidone and batimastat. The present study showed that LPS-induced neutrophil recruitment in BAL fluid was inhibited by 200 mg/kg pirfenidone. Sequestration of neutrophils in vascular, interstitial, and alveolar spaces is thought to damage the delicate structure of the lung by generating reactive oxygen species and releasing proteolytic enzymes mainly MMP-9 (Hibbs et al., 1985). Therefore, the concomitant inhibition of the LPS-induced increase in MMP-9 by 200 mg/kg pirfenidone may be related to the changes in BAL cell composition. Indeed, MMP-9 is secreted from preformed granules of neutrophils under the influence of LPS and chemotactic factors, a mechanism that does not require direct de novo synthesis of MMP-9 (Masure et al., 1991). It has been shown that, in mice chronically exposed to LPS (Corbel et al., 2001a) and in humans (Torii et al., 1997), the number of neutrophils in BAL fluid correlates with an increase in MMP-9 levels. It has been demonstrated that dietary intake of pirfenidone decreases the bleomycin-induced increase in superoxide dismutase activity in the lung, indicating a lower level of reactive oxygen species formation (Iyer et al., 1995). Moreover, pirfenidone was found to be an effective scavenger of reactive oxygen species generated in vitro (Iyer et al., 1998). These results suggested that the antifibrotic action of pirfenidone may be attributed to attenuation of inflammatory events by both the reduced influx of inflammatory cells into the lung and its ability to scavenge the reactive oxygen species generated by inflammatory cells.

Moreover, pirfenidone (200 mg/kg) inhibited the production of both TNF-α and TGF-β in BAL following LPS aerosol. This effect of pirfenidone could be attributed to attenuation of the inflammatory events by the reduced recruitment of inflammatory cells into the lung. A previous study (Cain et al., 1998) showed that injection of 100 or 200 mg/kg pirfenidone inhibits the induction of circulating TNF following a single injection of LPS. It is well known that TNF-α is able to stimulate the production of other cytokines in vitro and in vivo (Tracey and Cerami, 1993). These facts suggest that TNF-α plays a central role in the cascade of cytokine production. This possibility is supported by the fact that administration of anti-TNF-α antibodies is effective to lower the levels of other cytokines in LPS-injected baboons (Fong et al., 1989). Elevated levels of TGF-β in animal models of lung fibrosis, and in BAL fluids and lung tissues of patients suffering from pulmonary fibrosis, are well-documented (Khalil et al., 1989, 1991). Our results suggest that the beneficial effects of pirfenidone involve in part the suppression of TGF-β effects in the acute inflammatory phase.

In contrast, batimastat did not modify either inflammatory cell recruitment or MMP-9 profile in BAL fluids induced by aerosol of LPS. This result is consistent with results of a recent in vitro study showing that hydroxamic acid-based synthetic MMP inhibitors had no significant effect on fMLP (*n* formyl-MET-LEU-PHE)-stimulated neutrophil migration through the endothelial cells and associated basal lamina (Mackarel et al., 1999). The fact that MMP may not be involved in cell migration was strengthened by results of in vivo studies using MMP-9 knockout mice exposed to LPS (Betsuyaku et al., 1999) or to bleomycin (Betsuyaku et al., 2000). Indeed, the lungs of MMP-9 deficient mice showed bleomycin-induced alveolar inflammation comparable to that seen in MMP-9 wild type mice (Betsuyaku et al., 2000).

Two recent studies using zymography have shown that batimastat inhibits directly MMP-2 and MMP-9 but does not affect MMP secretion (Mäkela et al., 1999; Zervos et al., 1999). In the present experiments, zymography showed no modulation of the LPS-enhanced MMP-9 activity by batimastat. This result is in agreement with the number of neutrophils in the BAL fluid of mice treated with batimastat. Batimastat is a synthetic, low molecular weight MMP inhibitor with a collagen-mimicking hydroxamate structure which facilitates chelation of the zinc ion in the active site of MMPs (Wojtowicz-Praga et al., 1997). Moreover, batimastat binds reversibly to the zinc-binding region of MMPs (Brown, 1995). Since batimastat chelation is reversible, it is possible that batimastat is removed from MMP during zymography. The experiment in which batimastat was added to the incubation buffer during zymography clearly indicated the direct inhibitory effect of batimastat, but not of pirfenidone, on MMP-9 activity in BAL fluid from LPS-exposed mice.

Inhibition of LPS-enhanced TNF-α and TGF-β in the BAL fluid was observed with treatment of mice with batimastat, confirming that the MMP inhibitor preparation used was active in vivo. MMP inhibitors were reported to inhibit the TNF-α converting enzyme (TACE) (Gearing et al., 1994; McGeehan et al., 1994; Solorzano et al., 1997) and reduce production of TNF-α (Murakami et al., 1998). Therefore, batimastat not only blocks matrix-degrading enzymes but also interferes with the release of bioactive TNF-α (17 kDa) (Gearing et al., 1994). More recently, TNF-α converting enzyme (TACE) was identified as a membrane-bound metalloproteinase that contain A Disintegrin And Metalloproteinase (ADAM) domain (Maskos et al.,1998). The findings with MMP inhibitors reported here and earlier suggest that blocking the release of soluble active TNF-α may offer the capacity to reduce TNF-α-mediated shock that accompanies Gram-negative bacteremia or sepsis syndrome. Solorzano et al. (1997) demonstrated a reduction of mortality in an LPS-induced model of shock the ameliorative effect of a hydroxamate MMP-inhibitor. This decrease of mortality was attributed to inhibition of the cleavage of the 26 kDa membrane-bound TNF-α to soluble 17 kDa TNF-α by MMPs. However, our results suggest that MMPs are important effectors in the pathogenesis inhibition of the cleavage of membrane-bound TNF-α. The physiologic mechanisms that regulate latent TGF-β activation are not well understood. Proteolysis by plasmin, cathepsin, and other enzymes can activate latent TGF-β (Lyons et al., 1990). It is thus not excluded that batimastat inhibits a MMP which could be involved in latent TGF-β activation, explaining the decrease in active TGF-β in BAL from mice exposed to LPS and treated with batimastat.

In the present study, TIMP-1 was increased after LPS exposure, as shown by Pagenstecher et al. (2000). This increase was not modified, whatever the treatment, pirfenidone or batimastat. Any induction of TIMP is likely to diminish the level of MMP activity and reduce degradation of macromolecular components in the extracellular matrix. Moreover, TIMPs may affect the angiogenic process in several ways, i.e. by inhibiting endothelial cell migration, preventing MMP-mediated endothelial cell detachment, blocking the release of matrix-bound angiogenic factors, and preventing degradation of the extracellular matrix (Johnson et al., 1994). TIMP-1 could be involved in protecting the alveolar structure from uncontrolled degradation after acute injury.

Batimastat and pirfenidone, efficient inhibitors of bleomycin-induced pulmonary fibrosis in mice, do not have the same effect on inflammatory cell infiltration following acute lung injury. The fact that batimastat, a synthetic inhibitor of MMP activity, is without effect on inflammatory influx, emphasizes the important role of MMPs in the development of pulmonary fibrosis.

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