ORIGINAL RESEARCH

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Anti-inflammatory and anti-fibrotic effects of sirolimus on bleomycin-induced pulmonary fibrosis in rats

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Abstract

Purpose: Pulmonary fibrosis is a devastating disease with a poor prognosis. Although the diagnosis and pathophysiology of this disease have been better characterized over the past few years, there is no effective therapy for the disease. The aim of this study was to evaluate the anti-inflammatory and anti-fibrotic effects of sirolimus (SRL), which is a potential anti-fibrotic agent, by using bleomycin (BLM)-induced pulmonary fibrosis model in rats.

Methods: A single intra-tracheal injection of BLM (2.5 U/kg) was administered and sirolimus (2.5 mg/kg/day) was given orally, beginning either one day before (early SRL) or nine days after (late SRL) the BLM administration. The effect of SRL on fibrosis was studied by analysis of cytokine levels in BAL fluid, measurement of lung tissue hydroxyproline (HPL) content and histopathological examination.

Results: Both early and late SRL administrations caused a decrease in the levels of IL-13, PDGF-A and TGF- β 1 ($p=0.001$) and an increase in IFN- γ levels ($p=0.001$) in BAL fluid. Early and late SRL also caused a decrease in HPL content ($p=0.001$). Early sirolimus caused a significant decrease in fibrosis score ($p=0.001$), while late SRL did not.

Conclusion: Sirolimus was effective in BLM-induced pulmonary fibrosis model, especially in the early phases of the disease.

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Pulmonary fibrosis is a progressive and fatal disease with unknown etiology. The prevalence of the disease increases with age. The incidence is as high as 250 cases per 100,000 in the elderly population [1,2]. It is characterized by fibroproliferation, deposition of extra-cellular matrix in the lung parenchyma and tissue remodeling. Patients with pulmonary fibrosis are generally treated with corticosteroids and/or cytotoxic agents such as azathioprine and cyclophosphamide, despite their significant side-effects. The long-term survival with current treatment regimens is poor. The mean survival is only two to three years following diagnosis; therefore, novel drugs with better efficacy and tolerance are urgently needed for the treatment of pulmonary fibrosis.

Since the current data shows lack of benefit with anti-inflammatory strategies, there has been a shift towards the use of anti-fibrotic agents. Sirolimus (SRL), which is also known as rapamycin, is a macrolide produced from the bacteria *Streptomyces hygroscopicus*. It has antifungal and immunosuppressive properties. It has anti-proliferative effects on lymphoid and non-lymphoid cells by inhibiting cytokine and growth factor mediated cell signaling [3]. Sirolimus has also been successfully used in the treatment of hepatic fibrosis in a rat model [4].

Intra-tracheal instillation of bleomycin (BLM) in rats has been used as a widely accepted experimental model of pulmonary fibrosis for many years [5]. Pathological findings resulting from this model are very similar to those found in human pulmonary fibrosis. It is known that inflammation is followed by fibrosis in the BLM model [6-8]. Although a large number of anti-fibrotic drugs has been evaluated in experimental pre-clinical studies, results from these studies have not yet been translated into clinical practice. Most of the studies, describing beneficial anti-fibrotic agents in BLM model, used a preventive regimen [9]. Since these agents are usually administered one day before or immediately after BLM injection, they may exert their effect through anti-inflammatory and anti-fibrotic mechanisms. It is critical to differentiate the drugs that interfere with the inflammatory and early fibrogenic response from those that prevent advancement of fibrosis: the latter are likely much more important for clinical application. Patients with idiopathic pulmonary fibrosis (IPF) usually do not refer to a doctor or hospital until the fibrosis period, and this delay may explain the failure of these agents to successfully treat pulmonary fibrosis.

The aim of this study was to evaluate SRL as a potential antifibrotic agent by assessing its effects on common biochemical and histological endpoints of BLM-induced pulmonary fibrosis. We attempted to distinguish the anti-inflammatory and anti-fibrotic effects of SRL, which has previously been

shown that it can inhibit the development of BLM-induced fibrosis.

Methods

Animals

A total of 37 male Sprague Dawley rats, weighed 200 to 230g, were studied. The animals were maintained in a controlled environment and fed on rodent chow and tap water *ad libitum*. They were free of respiratory and other diseases. All procedures were approved by the Experimental Animal Ethics Committee of Selcuk University, Experimental Medicine Research and Application Center.

Experimental Model and Study Groups

Prior to surgery, all the animals were anesthetized with a mixture of xylazine (10 mg/kg, Rompun; Bayer AG, Leverkusen, Germany) and ketamine (70 mg/kg, Ketalar; ParkeDavis, Eczacibasi, Istanbul, Turkey), which was administered by intramuscular injection. After a small cervical skin incision and separation of strap muscles, the trachea was exposed and punctured with a 26-gauge needle for the administration of either 0.3 ml of sterile saline solution (0.9% NaCl) or 2.5 U/kg BLM sulphate (Bleocin-S, Nippon Kayaku Co., Ltd, Tokyo, Japan) dissolved in 0.3 mL sterile saline [10]. This dose of BLM was determined, from previous experiments, to create consistent biochemical and histological damage without mortality [11,12].

Sirolimus solution (Rapamune, Wyeth Pharmaceuticals Inc., Rouses Point, USA) (2.5 mg/kg/day) was administered by daily gavage, beginning either 1 day before (early SRL) or 9 days after (late SRL) intratracheal (IT) BLM administration. Control animals received distilled water (DW) by daily gavage.

Animals were randomly distributed into five weight-matched study groups: (1) Control group; (2) BLM group; (3) Early SRL group; (4) Late SRL group; and, (5) SRL control group. Details of the groups are shown in Table 1.

TABLE 1. Details of study groups

Groups	n	IT application	Oral application	SRL timing
Control group	7	SLN	DW	-
BLM group	8	BLM	DW	-
Early SRL group	7	BLM	SRL	-1. day
Late SRL group	8	BLM	SRL	9. day
SRL control group	7	SLN	SRL	-1. day

IT: Intratracheal, SRL: Sirolimus, SLN: Saline, DW: Distilled water, BLM: Bleomycin.

Rats were sacrificed by transection of the abdominal aorta under an overdose of pentobarbital (80 mg/kg, intraperitoneal) 24 days after IT bleomycin or saline instillation.

Spleen weights were measured to assess the immunosuppressive effect of SRL.

Bronchoalveolar Lavage Fluid Biochemical Analysis

After clamping the right main bronchus, bronchoalveolar lavage (BAL) fluid was obtained from the left lung. Lavage was done four times with 3 ml 0.9% NaCl. Analysis of interleukin (IL)-13, interferon (IFN)- γ , platelet-derived growth factor (PDGF)-A and transforming growth factor (TGF)- β 1 was performed with ELISA technique using commercially available kits. The Invitrogen Rat IL-13 (BioSource, Invitrogen, USA, Cat #KRC0132/KRC0131), Invitrogen Multispecies TGF- β 1 (BioSource, Invitrogen, USA, Cat #KAC1688/KAC1688), Rat IFN- γ (BenderMedSystems, Austria, Cat #BMS621) and Quantikine Mouse/Rat PDGF-AB (R&D Systems, USA, Cat #MHD00) kits were used for determination of these parameters in the BAL fluid.

Lung Tissue Hydroxyproline Content

The left lung was ligated and cut at the hilum, blotted on filter paper and snap frozen in liquid nitrogen. Subsequently tissues were stored in Eppendorf tubes at -80°C until biochemical analysis. Frozen tissue samples were weighed and then alkaline hydrolysis process was performed. Briefly, the tissue samples were taken in to the Eppendorf tubes including 1 ml of 2 N NaOH and the tubes were tightly closed with rubber stoppers and incubated in an autoclave at 120°C for one hour. After hydrolysis, the derivatization process was carried out and 50 μ L of samples were injected to the HPLC system (Shimadzu Prominence, Japan). Commercially available hydroxyproline (HPL) analysis kit (HPLC, Eureka, Italy) was used for the analysis and the measurements were performed with UV/VIS

FIGURE 1. Spleen weights of the study groups. BLM: Bleomycin, SRL: Sirolimus. (*: $p < 0.001$; **: $p < 0.01$).

detector at 495 nm. Lung tissue hydroxyproline contents were given as mg/g frozen tissue.

Histopathological Examination

Histopathological evaluation was carried out on the right lungs, which were not lavaged. The right lung was fixed by IT infusion of formalin at 25 cm hydrostatic pressure. Lung tissue was embedded in paraffin and lung sections were stained with haematoxylin and eosin or Masson's trichrome for examination by light microscopy. A pathologist (HT) examined the sections blindly. Ashcroft scoring system was used to estimate the severity of pulmonary fibrosis on a numerical scale [13].

Statistical Analysis

The results were expressed as means $\pm$ standard deviation. Statistical analysis was carried out by Kruskal Wallis variance analysis and Dunn's multiple comparison test as a posthoc test. The p values < 0.05 were considered statistically significant.

TABLE 2. IL-13, PDGF-A, IFN- γ and TGF- β 1 levels in BAL fluid.

Groups	IL-13 (pg/ml)	PDGF-A (pg/ml)	IFN- γ (pg/ml)	TGF- β 1 (pg/ml)
Control group	0.34 $\pm$ 0.12	13.25 $\pm$ 1.59	109.35 $\pm$ 46.92	46.14 $\pm$ 24.89
BLM group	20.67 $\pm$ 3.13*	151.70 $\pm$ 38.21*	9.41 $\pm$ 2.39**	259.75 $\pm$ 67.46*
Early SRL group	5.64 $\pm$ 1.78 [†]	29.69 $\pm$ 5.32 [†]	31.66 $\pm$ 1.92 [†]	132.43 $\pm$ 9.36 [†]
Late SRL group	10.37 $\pm$ 2.20 [†]	71.96 $\pm$ 15.85 [†]	27.08 $\pm$ 7.80 [†]	157.25 $\pm$ 7.74 [†]
SRL control group	1.31 $\pm$ 0.48*	20.80 $\pm$ 2.68*	102.38 $\pm$ 38.73	66.29 $\pm$ 15.88

BLM: Bleomycin, SRL: Sirolimus. *: compared to control group ($p = 0.001$); **: compared to control group ($p = 0.001$); [†]: compared to BLM group ($p = 0.001$); [‡]: compared to BLM group ($p = 0.001$)

TABLE 3. HPL contents and Ashcroft scores of the study groups.

Groups	HPL (mg/g)	Ashcroft score
Control group	0.25 ±0.80	0
BLM group	0.88 ±0.62*	3.63 ±1.59*
Early SRL group	0.51 ±0.08**	1.71 ±0.75**
Late SRL group	0.55 ±0.08**	2.13 ±0.83
SRL control group	0.26 ±0.06	0.14 ±0.37

HPL: Hydroxyproline, BLM: Bleomycin, SRL: Sirolimus. *: compared to control group ($p=0.001$); **: compared to BLM group ($p=0.001$)

Results

Efficacy of sirolimus

Spleen weights were measured after 24 days of daily gavage, in order to assess the immunosuppressive effect of SRL. Spleen weights were significantly decreased in all SRL-treated groups ($0.264 \text{ g} \pm 0.03$ for early SRL, $0.344 \text{ g} \pm 0.01$ for late SRL and $0.385 \text{ g} \pm 0.03$ for SRL control group) compared to the control group ($0.602 \text{ g} \pm 0.08$) (Figure 1).

Effect of sirolimus on IL-13, PDGF-A, IFN- γ and TGF- β 1 levels

Recovery of BAL fluids ranged from 82 to 90% and were not significantly different among the five groups. The concentration of profibrotic cytokines IL-13, PDGF-A, and TGF- β 1 in BAL fluid was higher in the BLM group than in the control group. In addition, IT BLM administration significantly decreased the BAL fluid levels of the antifibrotic cytokine, IFN- γ compared with the control group. Both early and late SRL administration significantly decreased the levels of IL-13, PDGF-A and TGF- β 1 and increased the levels of IFN- γ compared with the BLM group (Table 2).

It was noteworthy that, although there were no significant differences in IFN- γ and TGF- β 1 levels between control and SRL control groups, IL-13 and PDGF-A levels were significantly higher in the SRL control group compared with the control ($p=0.002$). Details are presented in Table 2.

Effect of sirolimus on HPL content

Lung HPL concentration, a marker of collagen deposition, increased at 24 days after the exposure to BLM ($p=0.001$). Both early and late administrations of SRL to IT BLM caused a significant decrease in HPL levels. On the other hand, administration of SRL had no effect on HPL levels in the IT saline-administered group (Table 3).

FIGURE 2. Histological examination of the anti-inflammatory and antifibrotic effects of sirolimus on bleomycin instilled lungs. Lung sections stained with haematoxylin-eosin (HE) or Masson's trichrome (MS) stain. (a) Normal lung tissues showing normal open alveoli and interalveolar space with normal terminal bronchi (HE×80) (b) Bleomycin-treated lung sections showing severe congestion and edema of interalveolar space, inflammation and thickened and collapsed alveoli with marked fibrosis (HE×80). (c) Bleomycin-treated lung sections showing extensive peribronchial and perialveolar collagen deposition (white arrow) that obliterates the alveolar spaces (MT×200) (d) Meloxicam prior to bleomycin showing mild inflammation, moderate edema and minimal fibrotic changes (HE×80).

Histopathologic assessment

Histological assessment of hematoxylin and eosin or Masson's trichrome-stained sections of the right lungs at 24 days showed that normal open alveoli and interalveolar spaces were present in the control group (Fig 2a). The administration of BLM resulted in inflammatory infiltration, thickening of alveolar walls (Fig 2b) and an increase in interstitial collagen deposition (Fig 2c). Pre-treatment with SRL before BLM showed mild inflammation of perialveolar tissues with moderate edema of interalveolar spaces and minimal fibrotic changes (Fig 2d).

In the semi-quantitative assessment of lung sections, BLM group had significantly higher scores compared to the control group ($p=0.001$). This increase in fibrosis score, induced by BLM, was significantly reduced with early SRL treatment ($p=0.001$); however, late administration of SRL was not as effective as early administration ($p>0.05$). The grades of fibrosis in the five groups are presented in Table 3.

Discussion

The results of our study showed that both early and late application of SRL attenuated lung collagen accumulation as shown by a reduction in the HPL content, reduced levels of IL-13, PDGF-A, TGF- β 1, and increased levels of IFN- γ in BAL fluid in BLM-induced pulmonary fibrosis. Despite these promising effects, only early application of SRL provided amelioration of histopathological scores: there was no significant difference in Ashcroft scores between late SRL and BLM alone groups.

In a recent study [6], it has been shown that expression of inflammatory mediator proteins, such as IL-1 α , IL-1 beta, IL-6 and IFN- γ , significantly increased during the first nine days after BLM administration and then subsequently decreased. After day nine, there was an increase in pro-collagen I gene expression, TGF- β 1 expression and collagen deposition. As a result, the authors hypothesized that there was a “switch” between an inflammatory and fibrotic phase of the model at around day nine. For that reason, SRL was administered both one day before and nine days after BLM instillation.

Sirolimus has been shown to have potent immunosuppressive properties, including anti-lymphoproliferative effects, inhibition of growth factor and cytokine-mediated signaling [14]. It is generally used to prevent rejection in solid organ transplantation [15,16]. Because of its potent anti-proliferative effects, it has been used in a case with progressive pulmonary fibrosis and resulted in a good clinical response [17]. The rapamycin analogue, SDZ RAD, has been also studied as a therapeutic agent in the BLM model of pulmonary fibrosis [18]. In that study, SDZ RAD was administered one day prior to intra-tracheal BLM instillation and the rats were sacrificed on day 14. The authors showed that SDZ RAD had a dramatic inhibitory effect on lung-collagen accumulation in the BLM model, as measured by HPL content, but that the histopathologic assessment did not confirm this finding. This study demonstrated that early phase SDZ-RAD decreased HPL without any effect on pathology (Ashcroft score of 2.5 for BLM group and 3.5 for SDZ-RAD group). Unlike in the previously published study, in this study, SRL was administered both in the early and late period and the animals were sacrificed on day 24. At the end of the study, HPL contents were lower in both early and late SRL groups. This effect was also documented by histopathologic assessment in the early SRL group but not in the late SRL group. Despite promising effects of both early and late SRL on lung HPL concentrations in our study, histopathological evaluation failed to show a significant difference in Ashcroft scores between late SRL and BLM groups. This result can be explained by the limitations of the Ashcroft scale in quantifying

fibrotic tissue in BLM-induced lung damage. It has been reported that the Ashcroft scoring system has a high variability due to several factors [19]. Intratracheal application of BLM can cause patchy fibrosis with all grades and therefore it is sensitive to sampling errors, and using 10-fold magnification objective for microscope scanning may cause assessment errors because of greater heterogeneity of fibrotic changes in each microscopic field. Quantification of fibrosis by biochemical assessment of HPL is, therefore, a more consistent method for examining the effects of antifibrotic agents in this model. The reduction obtained with both early and late SRL is promising.

The primary effector cells in pulmonary fibrosis are myofibroblasts. These cells are thought to arise from proliferation/differentiation of resident lung fibroblasts, epithelial-mesenchymal transition or recruitment of circulating fibrocytes. Various cytokines, growth factors and signaling pathways are able to mediate these events. TGF- β 1, PDGF-A, IL-13 and IFN- γ are among the most important mediators in the pathophysiology of pulmonary fibrosis. These cytokines are biochemical endpoints used in many studies for fibrosis evaluation. In the present study, an increase was found in the levels of TGF- β 1, PDGF-A and IL-13, and a decrease in levels of IFN- γ ; in line with the literature. TGF- β function is essential in fibrosis [20] as this cytokine drives epithelial-mesenchymal transition, fibroblast to myofibroblast differentiation and is the most potent inducer of extracellular matrix production. Overexpression of this potent profibrotic mediator causes progressive fibrosis without any significant inflammation [21]. Several preclinical studies have shown that inhibition of TGF- β signaling provides attenuation of fibrosis, suggesting that drug targeting on the TGF- β pathway could offer a useful therapeutic intervention in IPF [22,23]. PDGF is another important fibrogenic mediator that induces fibroblast chemotaxis, fibroblast proliferation and promotes fibroblast mediated tissue matrix contraction [24]. PDGF cause hyperproliferation of type-II alveolar epithelial cells, recruitment of fibroblasts and formation of fibroblastic foci, which are the hallmarks in the pathogenesis of pulmonary fibrosis [21]. PDGF inhibition is an effective strategy to attenuate lung fibrosis [25,26]. IL-13 is a T-helper type 2 cytokine that is also strongly profibrotic and can stimulate fibroblast collagen production independent of TGF- β [27]. Since the overall cytokine pattern in biopsies from IPF patients appears to be T-helper 2 type (IL-13, IL-4), than T-helper 1 type mice deficient for IL-13 are protected in experimental lung fibrosis and IL-13 has recently joined the ranks of profibrotic cytokines in the lung. The profibrotic effect of IL-13 in the lung is postulated to involve irreversible fibroblast activation, triggered either directly or indirectly

through TGF- β . IL-13 is also an important therapeutic target for pulmonary fibrosis [28,29]. IFN- γ is a known inhibitor of wound repair and has been shown to attenuate fibrosis in BLM-induced pulmonary fibrosis. IFN- γ limits fibroblast proliferation, differentiation and collagen synthesis by inhibiting TGF- β expression and activity; therefore, levels of IFN- γ must be reduced in the patients with IPF. Ziesche *et al.* [30] showed substantial improvements in the condition of a small number of IPF patients following 12 months of IFN- γ treatment and suggested that this effect was mediated through inhibition of TGF- β 1 and CTGF. In BLM-induced pulmonary fibrosis, there is a deficiency of IFN- γ and of IFN-inducible chemokines. In the present study, decreased levels of TGF- β 1, IL-13, PDGF-A, and increased levels of IFN- γ were observed in both the early and late SRL groups as compared with the BLM group. These findings provide further evidence for the antifibrotic effect of SRL and support potential roles of these mediators in pulmonary fibrosis.

An important limitation of this study was that pulmonary fibrosis was assessed only with biochemical and histological endpoints. Recent studies have demonstrated efficacy of invasive lung function analysis in a BLM-induced pulmonary fibrosis model and suggest that the decline in lung function associated with fibrosis may not correlate with biochemical and histological endpoints [31,32]. Lung function, as the readout in an experimental model, can more accurately reflect the human pathological conditions. Physiological findings, such as forced vital capacity (FVC), diffusing capacity for carbon monoxide, total lung capacity and 6 minute walk test are important predictors of survival in humans with pulmonary fibrosis, and serial FVC more directly correlate with survival than does the pathological pattern [33-36].

Recent interest has focused on the role of chemokines in recruiting fibrocytes to the injured lung. It has been reported that CXCR4/CXCL12 axis also has an important role in pulmonary fibrosis by recruitment of circulating fibroblasts [37]. In a recent study, Mehrad *et al.* [38] showed that rapamycin reduced the number of CXCR4-expressing fibrocytes in the peripheral blood and lung and reduced lung collagen deposition in the mouse model of BLM-induced pulmonary fibrosis. In another study, using a transgenic mouse model of pulmonary fibrosis caused by lung-specific expression of the epidermal growth factor receptor ligand, TGF- α , Korfhausen *et al.* [39] demonstrated that administration of rapamycin prevented both the initiation and the progression of established pulmonary fibrosis and associated alterations in lung mechanics.

Many cases of pulmonary toxicity secondary to SRL have been reported in recent years [40]. Even though most of these cases with dyspnea, cough, fever and hemoptysis have been reported as interstitial pneumonitis, they also cover SRL-related pulmonary toxicity such as organizing pneumonia, focal fibrosis and alveolar hemorrhage [41]. In this study, the SRL control group was found to have similar hydroxyproline levels and histopathological scores as compared to the control group; however, levels of IL-13 and PDGF-A in the SRL control group were significantly higher than in the control group. Although the increase in these profibrotic cytokines is still much lower compared with the values in the BLM group, this may be explained by pulmonary toxicity of SRL. As published reports [40] state that pulmonary toxicity in humans begins 2.5 to 12 months after SRL administration, the short period of our study may not be sufficient for pulmonary toxicity of SRL.

In conclusion, the effect of SRL on the commonly used BLM-induced pulmonary fibrosis model was evaluated in this study. With our study design, positive effects of sirolimus were found in a clinically relevant model in which inflammation decreased and was replaced by fibrosis. In addition to the antifibrotic effects of SRL, there are pulmonary toxicity risks that should not be disregarded.

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