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Preventive effect of irbesartan on bleomycin-induced lung injury in mice

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ABSTRACT

Background: Idiopathic pulmonary fibrosis is a specific form of chronic fibrosing interstitial pneumonia that is limited to the lung. Angiotensin receptor blockers (ARBs) and peroxisome proliferator-activated receptor (PPAR) γ ligands have anti-inflammatory and anti-fibrotic effects. We investigated the effects of irbesartan—an ARB with PPAR γ activity—on the development of bleomycin-induced pulmonary fibrosis in mice.

Methods: Lung injury was induced in imprinting control region (ICR) mice by intratracheal instillation of 2 mg/kg of bleomycin. The treatment group orally received 20 mg/kg of irbesartan for 5 consecutive days before instillation. The mice were sacrificed and were evaluated 14 days after bleomycin instillation.

Results: Irbesartan reduced the fluid content and hydroxyproline level in the lung and improved the pathological findings as indicated by the Ashcroft score. Total cell counts, the numbers of macrophages, neutrophils, and lymphocytes, and the levels of transforming growth factor (TGF) β1 and monocyte chemotactic protein (MCP) 1 in the bronchoalveolar lavage fluid (BALF) were decreased. Treatment with a PPARγ antagonist GW9662 reversed some of the effects of irbesartan.

Conclusions: The results of this study indicated that irbesartan attenuated the development of bleomycin-induced pulmonary fibrosis in mice by decreasing TGF-β1 and MCP-1 via blocking of ATI, by binding to CCR2b, and by PPARγ-mediated inhibition of inflammation.

Abbreviations: IPF, idiopathic pulmonary fibrosis; AT1, angiotensin type 1 receptor; TGF, Transforming growth factor ; BALF, bronchoalveolar lavage fluid; PPARs, peroxisome proliferator activated receptors; ARBs, angiotensin receptor blockers; CCR2b, chemokine C–C motif receptor b; MCP-1, monocyte chemoattractant protein 1; ICR, imprinting control region; GW9662, 2-chloro-5-nitrobenzanilide; MIP-1α, macrophage inflammatory protein-1α; TNF-α, tumor necrosis factor-α; PGF2α, prostaglandin F2α; ELISA, enzyme-linked immunosorbent assay; CTGF, connective tissue growth factor; JNK, JUN N-terminal kinase; ANG1-7, angiotensin 1-7; R × Rs, retinoid × receptors; PPREs, PPARγ response elements

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1. Introduction

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic fibrosing interstitial pneumonia that is limited to the lung. The etiology and pathogenesis of IPF are not known, and it remains a devastating disease with a 5-year mortality rate greater than 50%. Although several drugs have been used to attempt to treat IPF, an established treatment that definitely improves its outcome does not exist [1]. Thus, we await new therapies based on a new understanding of the pathogenesis of IPF.

Angiotensin II is a peptide that plays a crucial role in regulating blood pressure and sodium homeostasis. To date, 4 angiotensin receptors have been identified: angiotensin type 1 receptor (AT1), type 2 receptor, type 3 receptor, and type 4 receptor. The vast majority of angiotensin II actions are mediated via the AT1 receptor. It is widely accepted that AT1 is involved in organ fibrosis, and inhibition of AT1 can suppress fibrosis of the heart, kidney, and lung [2–5]. Transforming growth factor (TGF)-β plays a critical role in the pathogenesis of IPF and bleomycin-induced fibrosis. Furthermore, it was reported that AT1 antagonists simultaneously suppress the TGF-β1 level in bronchoalveolar lavage fluid (BALF) [3,4].

Peroxisome proliferator activated receptors (PPARs) are a family of ligand binding nuclear hormone receptors. The pleiotropic effects of PPARs include lipid and lipoprotein metabolism and adipogenesis, glucose homeostasis, cell cycle regulation, and cellular proliferation and differentiation. Three PPARs encoded by 3 separate genes have now been identified: α, β/δ, and γ. PPARγ plays a role in regulating cell differentiation and inflammation and is thus of high interest as a potential target for therapies for diseases involving dysregulated inflammation and/or differentiation. In hyperoxia-induced acute lung injury, PPARγ expression is dysregulated and PPARγ induction has an essential protective role [6]. PPARγ agonists also significantly reduce lung injury and fibrosis induced by bleomycin in mice [7].

Angiotensin receptor blockers (ARBs) were developed for the treatment of high blood pressure to antagonize increased angiotensin II-dependent vasoconstriction. In addition to their AT1 blocking properties, several ARBs function as partial agonists of PPARγ; for example, telmisartan, candesartan, losartan, and irbesartan serve as PPAR ligands *in vitro* [8]. Irbesartan also has the beneficial effects of binding to chemokine C–C motif receptor b (CCR2b) to block monocyte chemoattractant protein 1 (MCP-1) binding [9] and inducing adiponectin by PPARγ activation [10,11]. The present study was designed to investigate the effect of irbesartan, an ARB with CCR2b binding and PPARγ activity, on the development of bleomycin-induced lung injury in mice.

2. Materials and methods

2.1. Mice and reagents

All mice received humane care in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication 8523, revised 1985; http://www.nyu.edu/uawc/Forms/Guideexcerpts). The Ethics Committee for animal experiments of Niigata University, Niigata, Japan, approved the study protocol (June 4th, 2009, Niigata University Research #32). Specific 8-week-old, pathogen-free, male imprinting control region (ICR) mice were obtained from Japan SLC (Niigata, Japan) and housed in the animal facility of Niigata University. Bleomycin was purchased from Nippon Kayaku (Tokyo, Japan). Dainip pon Sumitomo Pharma Co., Ltd. (Osaka, Japan) kindly sup plied us with irbesartan. The treatment group orally received 20 mg/kg of irbesartan for 5 consecutive days before bleomycin instillation. The PPARγ selective antagonist, 2-chloro-5-nitrobenzanilide (GW9662), was purchased from Sigma (St Louis, MO, USA).

2.2. Experimental design to determine the activity of irbesartan as an inhibitory agent in the bleomycin model

An experimental design to determine the activity of irbesartan in the bleomycin model is shown in Fig. 1. We used ICR

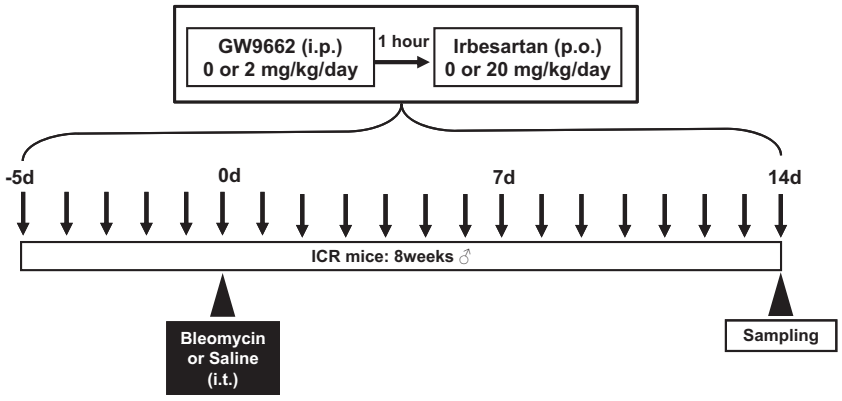

Fig. 1 – Experimental design to determine the activity of irbesartan in the bleomycin model. **Male ICR mice (8 weeks of age) were instilled intratracheally (i.t.) with bleomycin (2 mg/kg in 50 mL of saline) under anesthesia. The treatment group orally (p.o.) received irbesartan (20 mg/kg) for 5 consecutive days before bleomycin instillation. GW9662 (2 mg/kg) was injected intraperitoneally (i.p.) 1 h before irbesartan administration. The mice were sacrificed and evaluated 14 days after bleomycin instillation.**

mice because they are a well-characterized inbred strain susceptible to bleomycin-induced lung injury. To induce pulmonary fibrosis, we anesthetized the male ICR mice by intraperitoneal administration of 0.01 mL/g 10% pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL, USA) followed by intratracheal instillation of 2 mg/kg of bleomycin in 50 μl of sterile isotonic saline. The control animals received intratracheal saline only. The treatment group orally received 20 mg/kg of irbesartan for 5 consecutive days before bleomycin instillation in order to assess the toxicity of the agent simultaneously. GW9662 (2 mg/kg) was injected intraperitoneally 1 h before irbesartan administration. Because the changes in wet lung weight and BAL cell profiles exhibited similar results on days 7 and 14 in our preliminary experiments, we sacrificed and evaluated the mice 14 days after bleomycin instillation. Each experiment was repeated to confirm the findings, and the exact numbers of mice analyzed are shown on each figure panel in Section 3.

2.3. Histological analysis

The right lung was removed with the right main bronchus ligated and fixed by filling the lung through a tracheal cannula to 25-cm H_2O with 10% neutral buffered formaldehyde solution. The trachea was then occluded and fixation was continued for 4–10 days prior to the study. Longitudinal tissue sections of the lung were embedded in paraffin, stained with hematoxylin–eosin, and examined by light microscopy at a magnification of $\times 100$. Each histopathological experiment was performed in at least 3 or 4 mice per group. Visual grading of the pulmonary fibrosis was determined by Ashcroft's score [12]. Briefly, the entire field of each lung section was scanned at a magnification of $\times 100$, and each field was visually graded from 0 (normal lung) to 8 (total fibrotic obliteration of the field). The mean value of the grades obtained for all of the fields was used as the visual fibrotic score.

2.4. Measurement of hydroxyproline and fluid content of the lung

Hydroxyproline content (μg/lung) in the left lung of each subject was assayed according to the commonly used colorimetric measurement procedure (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Tokyo, Japan). The lungs were harvested and homogenized in 1 mL of PBS (pH 7.4) with a tissue homogenizer. A 1.5-mL volume of each sample was then digested in 1 mL of 6 N hydrochloric acid for 8 h at 120 °C. Citrate–acetate buffer (5 μl; 5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid, pH 6.0) and chloramine-T solution (100 mL; 282 mg of chloramines-T, 2 mL of n-propanol, 2 mL of H_2O, and 16 mL of citrate–acetate buffer) were added to each 5-mL sample and the samples were left at room temperature for 20 min. Next, 100 mL of Ehrlich's solution, 9.3 mL of *n*-propanol, and 3.9 mL of 70% perchloric acid were added to each sample, and the samples were incubated for 15 min at 65 °C. Samples were cooled for 10 min and read at 550 nm on a spectrophotometer. Hydroxyproline concentrations from 0 to 400 mg/mL were utilized to construct a standard curve.

The wet lung weight of the left lobe was measured after careful excision of extraneous tissues. The lung was exposed for 48 h at 180 °C and the dry weight was measured. Water content was calculated by subtracting the dry weight from the wet weight.

2.5. BALF sampling

BAL was performed 4 times by applying a tracheal cannula to the right lung with 0.5 mL of saline, with the left main bronchus ligation. Approximately 1.8 mL (90%) of BALF was recovered from each mouse examined. A 100-μl aliquot was used for the total cell count, and the remainder was immediately centrifuged at 1000*g* for 10 min. The total amount of cells was counted by using a hemocytometer, and cell differentiation was determined for more than 500 cells on cytocentrifuge slides with Wright–Giemsa staining. The BALF supernatants were stored at −80 °C prior to use.

2.6. Assays for proinflammatory cytokines in the BALF

MCP-1, TGF-β1, macrophage inflammatory protein-1α (MIP-1α), tumor necrosis factor-α (TNF-α), and prostaglandin F2α (PGF2α) were measured in the BALF by enzyme-linked immunosorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, USA for MCP-1, TGF-β1, MIP-1α, and TNF-α; Cayman Chemical, Ann Arbor, MI, USA for PGF2α).

2.7. Statistical analysis

Data are expressed as mean ± SEM. For multiple comparisons, we performed a one-way analysis of variance and Fisher's protected least-significant differences method was used as a post-hoc test. p Values of <0.05 were considered statistically significant.

3. Results

3.1. Effects of irbesartan on body weight

We measured the body weight of the mice at 5 days and found that pre-administration of the drug for 5 days did not cause a significant change in body weight (data not shown). The severe lung injury induced by bleomycin administration caused loss of body weight, and the body weight of the animals was monitored on day 14 to assess the protective effects of irbesartan. Irbesartan treatment inhibited weight loss and treatment with GW9662 reversed the inhibitory effect of irbesartan on weight loss (Fig. 2).

3.2. Effects of irbesartan on fluid and hydroxyproline contents of the lung

Bleomycin injection elicited an inflammatory response characterized by the accumulation of water in the lung as indicated by fluid content. Treatment with irbesartan significantly reduced the fluid content on day 14 after bleomycin instillation (Fig. 3A). GW9662 addition did not inhibit this reduction. Additionally, irbesartan treatment significantly

decreased the hydroxyproline content of the lung (Fig. 3B). These findings indicate that irbesartan administration may be effective in preventing bleomycin-induced lung fibrosis.

3.3. *Effects of irbesartan on histopathologic findings*

To evaluate the anti-fibrotic effect of irbesartan, mice treated with 2 mg/kg of bleomycin with or without irbesartan were histologically evaluated on day 14 after bleomycin instillation. We observed bleomycin-induced alveolitis and patchy fibrosis with destruction of the alveolar structure mainly in the subpleural regions (Fig. 4B); all of these symptoms were improved by 20 mg/kg of oral daily irbesartan (Fig. 4C). Administration of GW9662 reduced the beneficial effect of irbesartan (Fig. 4D). The microscopic findings were scored as described in the Materials and Methods, revealing that irbesartan administration significantly decreased the pathological grade of inflammatory cell infiltration and pulmonary fibrosis on day 14 after the bleomycin challenge (Fig. 4E).

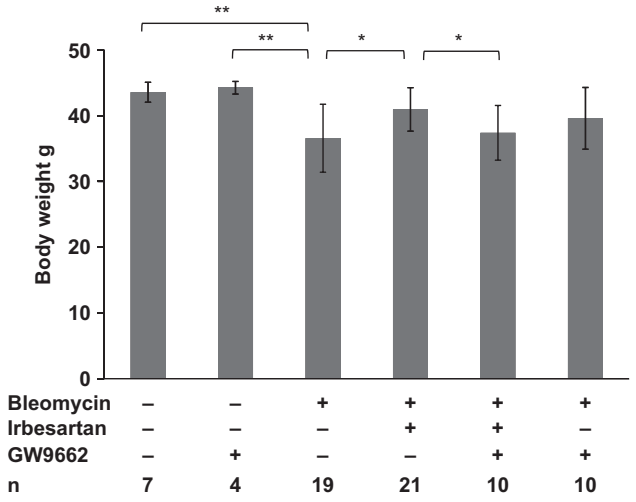

Fig. 2 – Effects of irbesartan on the body weight of bleomycin-treated mice. The body weight of animals was monitored on day 14. Although the irbesartan treatment group was significantly heavier than the bleomycin group, weight loss was not reduced in the GW9662 (PPARγ antagonist)-administered group. $^{*}p<0.05$ and $^{**}p<0.01$.

3.4. *Effects of irbesartan on BALF cell analysis*

We analyzed the cells in the BALF to evaluate the effects of irbesartan on the inflammatory responses induced by bleomycin. The total cell counts and the number of macrophages, neutrophils, and lymphocytes were significantly lower in the irbesartan treatment group than in the bleomycin group (Fig. 5). The effects of GW9662 varied according to the specific measure.

3.5. *Effects of irbesartan on proinflammatory cytokine concentration in the BALF*

On day 14, the TGF-β and MCP-1 levels in the BALF were higher in the bleomycin group than in the vehicle-treated group. Irbesartan significantly decreased the levels of TGF-β and MCP-1 in the BALF (Fig. 6). However, the MIP-1α, TNF-α, and PGF2α levels were not different between the irbesartan group and the bleomycin group (data not shown).

4. Discussion

The results of our study show that irbesartan attenuated the development of bleomycin-induced pulmonary fibrosis in mice by decreasing the TGF-β1 and MCP-1 levels in the BALF. Only 2 variables, body weight and hydroxyproline content, were significantly different between bleomycin/irbesartan-treated mice and bleomycin/irbesartan/GW9662-treated mice, whereas most of the other variables showed a higher value

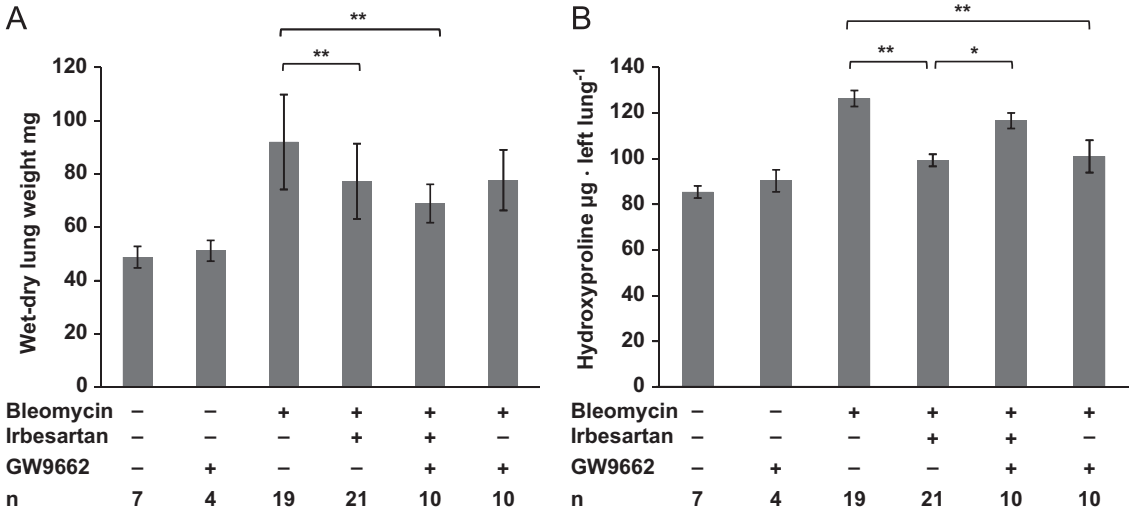

Fig. 3 – Effects of irbesartan on water accumulation and the hydroxyproline content of the lung. The accumulation of water (A) and hydroxyproline content (B) in the lung were significantly increased by bleomycin injection, and both were significantly attenuated by irbesartan administration. GW9662 reversed the effect of irbesartan on hydroxyproline content (B). $^{*}p<0.05$ and $^{**}p<0.01$.

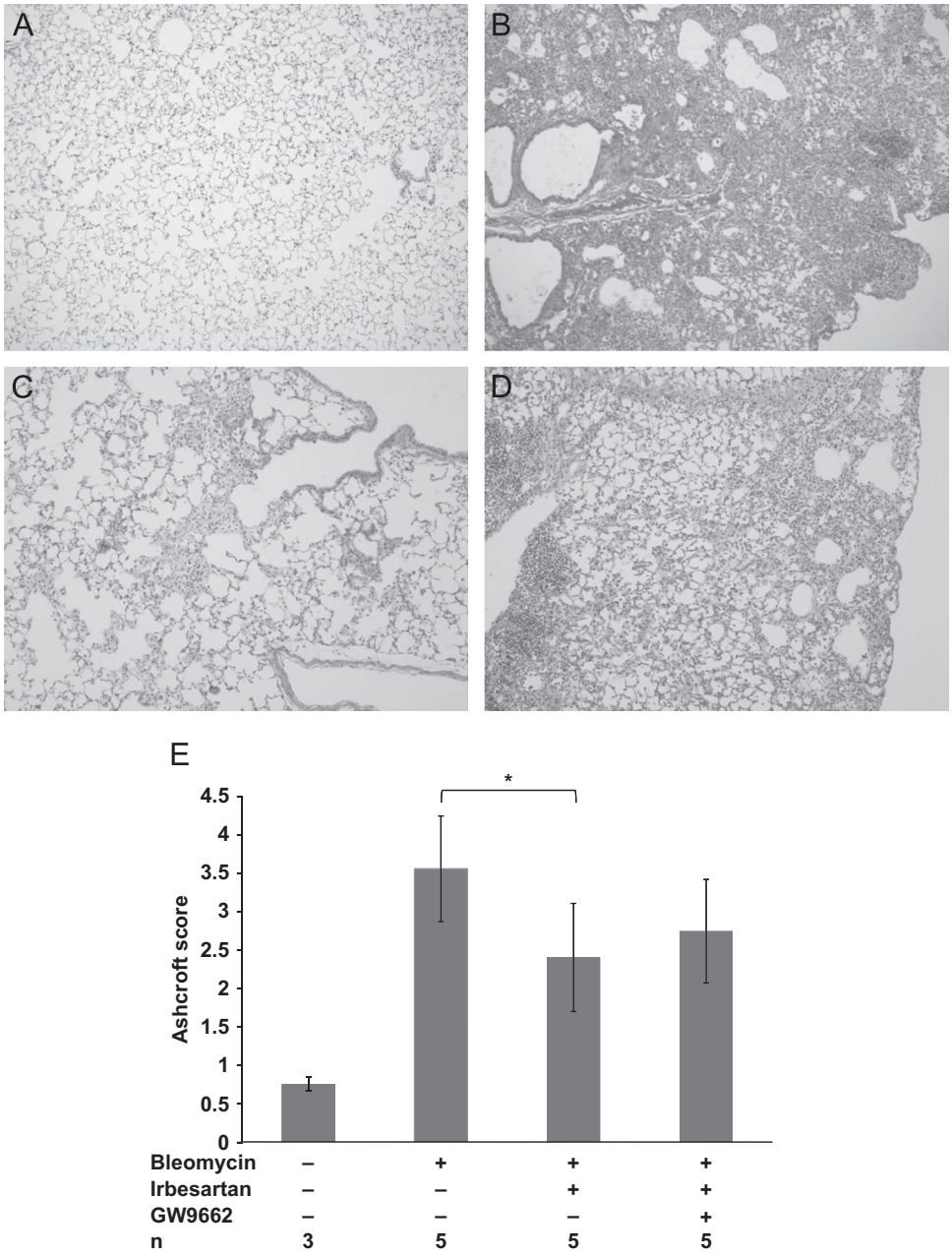

Fig. 4 – Effects of irbesartan on histopathologic findings. **On day 14 after the bleomycin challenge compared with control (A, ×100), alveolitis and patchy fibrosis with destruction of the alveolar structure were observed mainly in the subpleural regions (B, ×100) ; irbesartan ameliorated these changes (C, ×100). GW9662 administration reduced the beneficial effect of irbesartan (D, ×100). We visually graded the pulmonary fibrosis by Ashcroft's score. Irbesartan significantly decreased Ashcroft's score, suggesting less severe pulmonary fibrosis. GW9662 modestly reversed the effect of irbesartan on the Ashcroft score (E). *$p < 0.05$.**

in the latter group. Although pathological differences were apparent between the 2 groups, the Ashcroft score did not show a significant difference; this was probably because of low sample numbers and the blocking of CCR2b/MCP-1. Because GW9662 is a PPARγ-selective antagonist, we concluded that irbesartan partially attenuated the development of bleomycin-induced pulmonary fibrosis in mice through PPARγ-mediated inhibition of inflammation.

Pulmonary fibrosis is pathologically characterized by the features of persistent and unrepaired epithelial damage, proliferation and accumulation of fibroblasts and myofibroblasts, and increased collagen deposition. Angiotensin II is now recognized as a growth factor that regulates cell growth and fibrogenesis. Alveolar epithelial cells and myofibroblasts synthesize angiotensin II in the human fibrosing lung. It increases expression of TGF-β1 [13], connective tissue growth factor (CTGF) [14], and type I collagen, which is a principal matrix protein in the lung interstitium by lung fibroblasts [13]. Angiotensin II induces type I collagen synthesis by AT1 receptor activation and via the autocrine action of TGF-β1

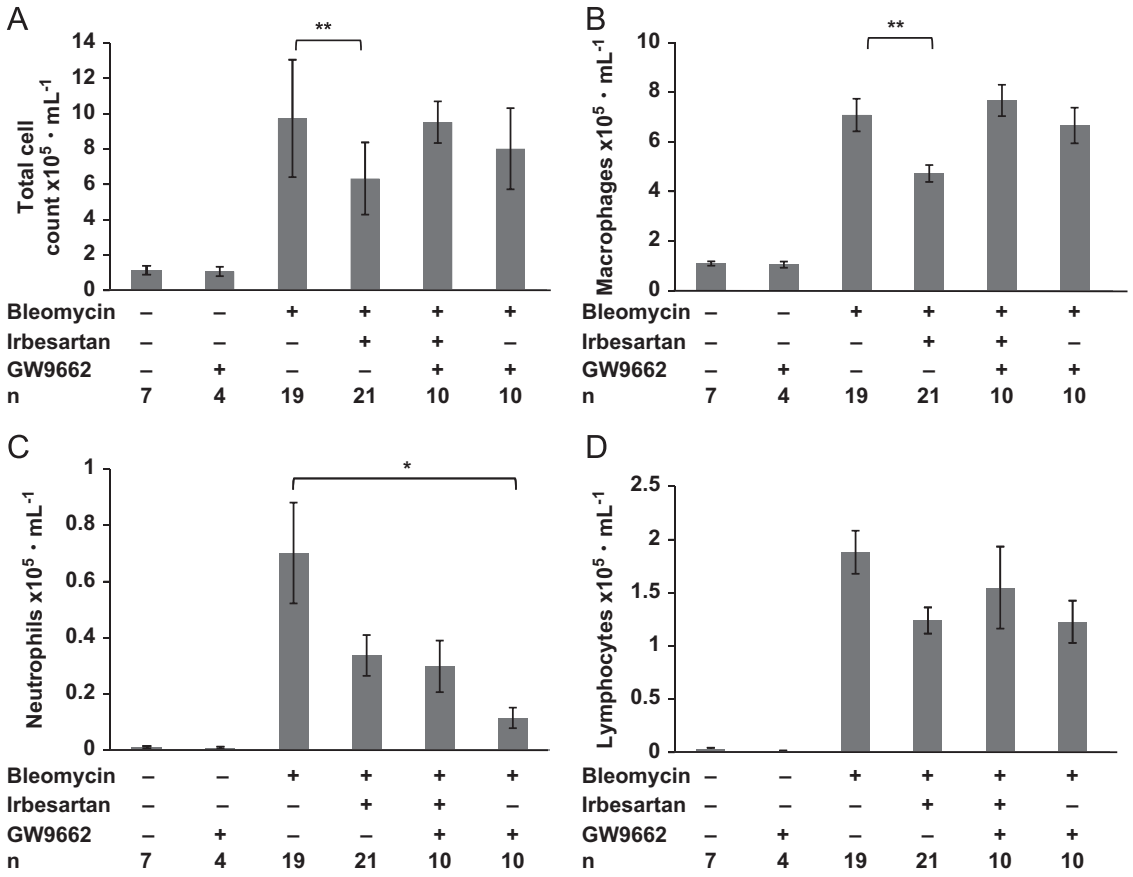

Fig. 5 – Effects of irbesartan on BALF cell analysis on day 14. Irbesartan significantly decreased the total cell count (A) and the numbers of macrophages (B), neutrophils (C), and lymphocytes (D) in the BALF of bleomycin-induced lung fibrosis. The effects of GW9662 on these values were not consistent. $^*p<0.05$ and $^{**}p<0.01$.

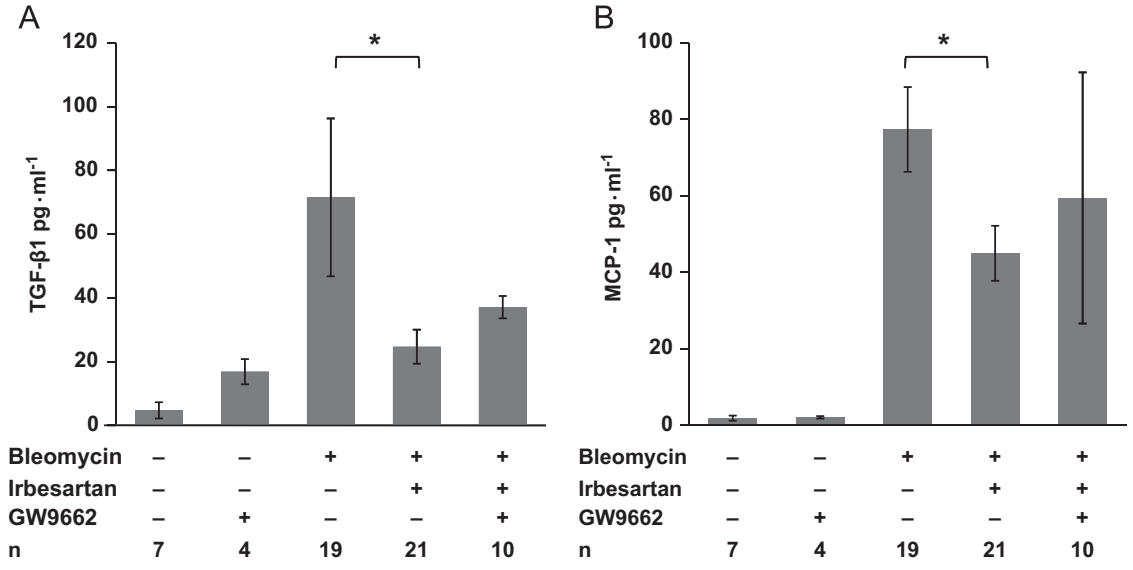

Fig. 6 – Effects of irbesartan on proinflammatory cytokine concentrations in the BALF on day 14. Irbesartan significantly decreased the levels of TGF-β1 (A) and MCP-1 (B) in the BALF of bleomycin-induced lung fibrosis. However, it did not change the levels of MIP-1α, TNF-α, and PGF2α (data not shown). $^*p<0.05$.

and CTGF. It also has profibrotic actions on migration and myofibroblast transition mediated through both AT1 and AT2 receptors on lung fibroblasts. Apoptosis of alveolar epithelial cells is one of critical events in the pathogenesis of pulmonary fibrosis [15–17], and angiotensin II induces apoptosis of alveolar epithelial cells through the AT1 receptor [18]. The

phosphorylation of JUN N-terminal Kinase (p-JNK) is required in alveolar epithelial cell apoptosis in response to angiotensin II or bleomycin. Angiotensin converting enzyme-2 produces angiotensin 1-7 (ANG1-7), which inhibits apoptosis by blocking JNK phosphorylation through the ANG1-7 receptor *mas* [19]. Thus, in addition to ARBs, ANG1-7 and ANG1-7 mimics, *mas* activators, or other JNK phosphorylation inhibitors could have the potential to inhibit both lung epithelial apoptosis and subsequent fibrosis.

Irbesartan induces PPARγ activity independent of the AT1R [10], but its activity as a modulator of PPARγ is lower than that of telmisartan or losartan [9]. GW9662 markedly inhibited irbesartan-induced adiponectin expression by blockade of PPARγ activation [11]. Taken together, these data clearly indicate that irbesartan has PPARγ agonistic activity and that GW9662 can block this action. Although we did not find inhibition of irbesartan activity in wet–dry lung weight differences and BALF neutrophils, hydroxyproline was significantly higher in bleomycin/irbesartan/GW9662-treated mice. Because the wet–dry lung weight indicates the fluid content of the inflamed lung, our results suggest that the acute inflammatory phase associated with PPARγ activation had ended, resulting in higher accumulation of hydroxyproline, on day 14 in this model. Neutrophils account for a small fraction of BAL cells, particularly in the late phase of the bleomycin model, which may explain why the effects of GW9662 were not remarkable. In the classical PPARγ-dependent pathway, PPARs form a heterodimer with retinoid × receptors (R × Rs), bind to PPARγ response elements (PPREs), and coordinately regulate cell differentiation and inflammation. PPARγ ligands also exhibit direct effects that do not involve transcriptional activation by the PPAR–R × R transcriptional complex. These direct effects may involve the interaction of the PPARγ protein with PPARγ ligands without the involvement of the R × R or PPRE, or may be completely independent of PPARγ [20,21]. PPARγ independent effects can alter multiple cellular programs including regulation of differentiation, inflammation, and apoptosis as well as anti-fibrotic activity [22–25]. Thus, many PPARγ ligands are under investigation as potential targets for therapies for human diseases.

The addition of GW9662 to a bleomycin-induced lung injury model in mice caused significant changes in the hydroxyproline content and neutrophils in the BAL. Because GW9662 is a PPARγ-selective antagonist, these results indicate that internal PPARγ activation might occur in this model, which has not yet been shown. However, the addition of GW9662 to a bleomycin-induced lung injury model treated by irbesartan resulted in increased hydroxyproline, suggesting that internal PPARγ activation functions in a different manner than the external PPARγ agonist—irbesartan.

We demonstrated that the MCP-1 concentration in the BALF was significantly decreased in bleomycin/irbesartan mice and GW9662 did not reverse this effect completely, suggesting that other mechanisms attenuate the development of bleomycin-induced pulmonary fibrosis. Irbesartan exhibits a strong affinity for CCR2b, the receptor for MCP-1. Since MCP-1 is a potent chemoattractant of monocytes, the CCR2/MCP-1 axis is essential in various inflammatory diseases involving monocyte/macrophage recruitment. MCP-1 expression is increased in the lungs of patients with IPF [26]. MCP-1 levels in the serum and BAL correlate with the clinical course of interstitial lung disease treated with corticosteroid therapy and the severity of lung injury in acute respiratory distress syndrome, respectively [27,28]. Because the production of MCP-1 is increased in an autocrine manner, irbesartan might work as a CCR2b blocking agent, reducing MCP-1 production, in addition to its activity as a PPARγ ligand. The reduced MCP-1 may attenuate bleomycin-acute lung injury in part by the mechanism proposed by Saito et al. [29].

We demonstrated in this study that irbesartan reduced weight loss, lung fluid and hydroxyproline contents, and the number of total cells, macrophages, neutrophils, lymphocytes, and MCP-1 and TGF-β levels in the BALF and improved outcome in histopathologic findings in a bleomycin-induced lung injury model. The addition of GW9662, a PPARγ antagonist, inhibited the irbesartan-induced reduction in weight loss and lung hydroxyproline contents of the left lung, but this inhibition was not observed in the lung fluid contents. Histological findings showed that GW9662 administration reduced the beneficial effect of irbesartan. Because the histological changes were direct evidence of the effects of irbesartan or GW9662, our results suggest that irbesartan has a strong therapeutic potential for bleomycin-induced lung injury, which is partially due to PPARγ-mediated inhibition of inflammation. Since the bleomycin model is mainly characterized by acute inflammation, most of the anti-fibrotic effects in the study might be a result of PPARγ-mediated inhibition of the inflammation that precedes fibrosis. We administered irbesartan 7 days after bleomycin instillation in preliminarily experiments, and we did not find significant improvement of bleomycin-induced lung injury. Although IPF was once thought to be a chronic inflammatory process, current evidence indicates that abnormally activated alveolar epithelial cells drive the fibrotic response with little inflammation [30]. Thus, it will be necessary to investigate the possible effects of irbesartan in fibrosis models without overt inflammation.

5. Conclusion

The results of the present study suggest that irbesartan inhibits bleomycin-induced lung injury and fibrosis by blocking ATI and binding to CCR2b, and via PPARγ-mediated inhibition of inflammation. Additional experimental research on pulmonary fibrosis without significant inflammation and clinical studies on other interstitial lung diseases such as acute exacerbation of IPF, interstitial lung diseases associated with collagen vascular diseases, or chemotherapy related toxicity are needed.

Conflict of interest

The authors have no potential conflicts of interest.

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