Protection against bleomycin-induced lung injury by IL-18 in mice

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PULMONARY INFLAMMATION and fibrosis are caused by drugs, infection, sepsis and radiation. Bleomycin (BLM)-induced lung injury has become a model for interstitial pneumonitis and pulmonary fibrosis (2). Patients with fibrotic diseases show alveolar epithelial cell injury and hyperplasia, deposition of collagen, and scar formation in the lung (10, 16). It has been shown that cytokines and reactive oxygen species (ROS) are involved in the acute lung injury of pulmonary fibrosis (17, 34, 37). In the normal airway, antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase, and catalase, protect the mucosa from various oxidative stresses (36). However, the pathophysiological mechanism for the lung injury still remains obscure.

BLM is an antineoplastic drug commonly used for the treatment of various carcinomas and lymphomas. It causes toxic side effects on lung tissue in ~3–5% of patients. Although the number of affected patients is small, the damage incurred by BLM in these patients is often irreversible and, at times, fatal (40). Studies of BLM-induced pneumonitis in animals have shown that proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, IL-6, and IL-8, are involved in interstitial pneumonia (4, 6, 18), and growth factors like transforming growth factor-β and platelet-derived growth factor are mainly responsible for ensuing fibrosis (28). On the other hand, granulocyte/macrophage-colony stimulating factor (GM-CSF) and IL-10 have been shown to suppress BLM-induced lung injury by inducing prostaglandin E2 (21) and suppressing proinflammatory cytokines (19), respectively. In addition, SODs have been reported to reduce damages caused by BLM (38). It has been reported that IL-1 pretreatment protects mice from BLM-induced pneumonitis by inducing Mn-SOD at the acute phase. Induction of Mn-SOD by IL-1 occurs quickly, within 6 h, resulting in suppression of pulmonary fibrosis (39).

IL-18 is a proinflammatory cytokine of the IL-1 family that was first identified as an IFN-γ-inducing factor (24). Recent studies have revealed that IL-18 also augments the production of anti-inflammatory or Th2 cytokines, such as IL-4, IL-10, and IL-13 (22). Moreover, IL-18 is expressed not only in immune cells but also in skin keratinocytes (32), intestinal epithelial cells (35), and airway epithelium (3). In this study, we examined the role of IL-18 in the regulation of inflammatory tissue injury caused by BLM. We also investigated the effect of pretreatment of mice with IL-18 on BLM-induced lung injuries.

MATERIALS AND METHODS

All animal experiments were approved by Experimental Animal Welfare Committee in Hyogo College of Medicine.

Animals. Six-week-old female C57BL/6 mice were purchased from Seac Yoshitomi (Fukuoka, Japan). IL-18 knockout (IL-18−/−) mice of the C57BL/6 background were kindly given by Dr. K. Nakanishi of our institute. The mice were kept in air-conditioned rooms at 25 ± 2°C and given tap water and solid food (MF; Charles-River Japan, Tokyo, Japan) ad libitum.

Intratracheal BLM instillation. Mice were anesthetized with ketamine hydrochloride (80 mg/kg im; Sankyo, Tokyo, Japan), and a 1-cm midline cervical incision was made to expose the trachea. Intratracheal instillation of BLM (2 mg/kg; Nippon Kayaku, Tokyo, Japan) or vehicle (sterile isotonic saline solution), in a volume of 1.5-ml/kg, was performed via a microliter syringe with a bent 26-gauge needle (Hamilton, Reno, NV). The cervical incision was closed with suture immediately after intratracheal instillation. The animals recovered quickly after surgery.

Treatment of mice with IL-18. Recombinant mouse IL-18, kindly donated by GlaxoSmithKline Pharmaceuticals, was diluted with sterile saline containing 0.5% mouse serum and injected into the peritoneal cavity at a dose of 2 μg/mouse “·day”−1 6 and 3 days before BLM instillation (two injections in total).

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Analysis of hydroxyproline content in the lung. At 21 days after BLM instillation, mice were killed with ether, and the lungs were removed. To measure hydroxyproline content, the left lung was excised, freeze-dried, hydrolyzed in 2 ml of 6 M HCl containing 0.01% (vol/vol) phenol at 110°C for 24 h, and dried at 50°C. The sample was dissolved in 1 ml of 0.02 M HCl and filtered through a disc filter of 0.45-μm pore size (Nihon Millipore, Tokyo, Japan). The amount of hydroxyproline in 10 μl of the filtrate solution was measured by a high-speed amino acid analyzer (L-8500, Hitachi, Tokyo, Japan). The hydroxyproline content in the lung was expressed as μg/g of wet tissue.

Histological assessment of the lung. At 7 and 21 days after BLM instillation, the right lung of each animal was fixed with 4% of neutralized phosphate-buffered paraformaldehyde (Hayashi Pure Chemical Ind., Osaka, Japan) and paraffin embedded. The tissues were cut into 5-μm sections and stained with hematoxylin and eosin for morphological analysis.

Analysis of cell numbers in bronchoalveolar lavage fluid. At 7 and 14 days after BLM instillation, mice were killed with ether, and the bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile saline five times. About 4 ml of BALF was routinely recovered from each animal. Cells were stained with trypan blue and counted with a hemocytometer.

Myeloperoxidase activity in BALF. BALF cells were collected by centrifugation at 400 g for 5 min, resuspended in 0.1 M K2HPO4 buffer, sonicated for 90 s, and centrifuged at 12,000 g for 10 min. To measure myeloperoxidase (MPO) activity, the supernatant (0.35 ml) was mixed with 0.3 ml of Hanks’ balanced saline solution containing 0.25% bovine serum albumin, 0.25 ml of 0.1 M K2HPO4 (pH 7.0), 0.05 ml of 1.25 mg/ml of o-dianisidine (Sigma-Aldrich, St. Louis, MO), and 0.05 ml of 0.05% H2O2 and incubated at 25°C for 10 min. The reaction was terminated by adding 0.05 ml of 1% NaN3, and absorbance at 460 nm was measured using a Beckmann DU-7500 spectrophotometer (Beckmann Coulter, Fullerton, CA).

Analysis of IL-10 and GM-CSF in the lung. The lung was homogenized with ice-cold PBS containing 0.5% Nonidet P-40 and protease inhibitors (Complete mini; Roche Diagnostics, Mannheim, Germany) and centrifuged at 12,000 g. The supernatant was removed and analyzed for IL-10 and GM-CSF using enzyme-linked immunosorbent assay kits (BD-PharMingen).

RT-PCR analysis of Mn-SOD mRNA. Total messenger RNA was extracted from the lung using ISOGEN (Nippon Gene, Tokyo, Japan). The reverse transcription reaction and the subsequent PCR were carried out with a RT-PCR kit (Applied Biosystems) using the following primers and amplification conditions: Mn-SOD (sense 5′-GACCTGCTTAC-GACTATGG-3′, anti-sense 5′-GACCTTGCTTATGAAAGC-3′, 94°C for 30 s, 58°C for 1 min, 72°C for 1 min, 35 cycles); β-actin (sense 5′-GTAGCGATATCGCTGGCCTG-3′, anti-sense 5′-GTACGAC-CAGAGGATACAGG-3′, 94°C for 30 s, 58°C for 1 min, 72°C for 1 min, 35 cycles). PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Measurement of SOD activity in the lung. Mice were treated with BLM or vehicle and, 24 h later, killed, and perfused with PBS to remove erythrocytes from the lung. The lung was excised, homogenized using Polytron homogenizer (Kinematica, Littau, Switzerland), and centrifuged at 10,000 g for 30 min. The supernatant was removed and analyzed for SOD activity using Bioxytech SOD-525 (Oxis Research).

Statistical analysis. Statistical analysis was performed using a computer program, StatView II (Hulinks, Tokyo, Japan), and a P value <0.05 was considered significant.

RESULTS

Comparison of mortality and lung hyperplasia caused by BLM between wild-type and IL-18−/− mice. To study the effect of IL-18 on the severity of lung injury by BLM, we compared the BLM-induced mortality between wild-type and IL-18−/− mice. As shown in Fig. 1, 95 and 80% of wild-type mice survived at 12 and 17 days after BLM instillation, respectively. In contrast, 50 and 40% of IL-18−/− mice survived at 10 and 17 days after BLM instillation, respectively. The histological evaluation of the lung at 7 days after BLM instillation showed that wild-type mice contained focal pneumonic lesions mainly in subpleural, perivascular, and peribronchial areas. In contrast, IL-18−/− mice developed diffused pneumonic lesions with loss of normal alveolar architecture and increased infiltration of inflammatory cells in peribronchial areas (Fig. 1B). We determined the Ashcroft score (21a) to evaluate morphologic data. The results showed that the lung injury in wild-type mice was much less than that in IL-18−/− mice.

Comparison of the effect of BLM on inflammatory cell infiltration and MPO activity in BALF between wild-type and IL-18−/− mice. We performed kinetic analysis of inflammatory cell invasion and MPO activity in BALF in wild-type and IL-18−/− mice instilled with BLM. In wild-type mice, the number of total infiltrated leukocytes increased 2.5-fold at day 7 and remained at that level at day 14 (Fig. 2A). In IL-18−/− mice, the number of total infiltrated cells increased 3.5-fold at...
day 7 and 4.8-fold at day 14 (Fig. 2A). All three types of infiltrating cells, lymphocytes, macrophages, and neutrophils, increased in a similar pattern (Fig. 2A). MPO activity in BALF was significantly higher in IL-18−/− mice than in wild-type mice at day 7 (Fig. 2B). These results suggest that BLM-induced lung injuries were exacerbated by the deficiency of IL-18.

Comparison of the effect of BLM on GM-CSF and IL-10 content and SOD activity in the lung between wild-type and IL-18−/− mice. Several groups have shown that GM-CSF, IL-10, and SOD play protective roles in BLM-induced pneumonitis (2, 20, 21). In this study, we analyzed levels of GM-CSF and IL-10 and activity of SOD in the lung of wild-type and IL-18−/− mice 7 days after BLM instillation. BLM instillation decreased GM-CSF levels in IL-18−/− mice more than in wild-type mice (69.0 vs. 57.1% reduction, P < 0.05, Student’s t-test; Fig. 3A). BLM caused reduction of IL-10 content in IL-18−/− and wild-type mice to similar degrees (58.2 vs. 57.0% reduction) (Fig. 3B). BLM instillation decreased SOD activity in the lung in IL-18−/− mice more than in wild-type mice (62.2 vs. 33.4% reduction, P < 0.05, Student’s t-test; Fig. 3C). These results suggest that IL-18 is protective against BLM-caused lung injuries in mice.

Effect of pretreatment with IL-18 on the BLM-induced mortality and lung hyperplasia. We examined whether treatment of IL-18 alters the mortality caused by BLM in wild-type mice. First, we administered IL-18 with BLM and found that this had no effect on the mortality. Next, we examined the effect of prophylactic administration of IL-18 by injecting recombinant mouse IL-18 (2 μg/mouse) intraperitoneally at 6 and 3 days before BLM instillation (two injections in total). This resulted in survival of all the mice at day 21, compared with survival of 80% of mice not treated with IL-18 (Fig. 4A). Similarly, pretreatment with IL-18 resulted in no death of IL-18−/− mice by BLM instillation (data not shown). Histological analysis showed pretreatment with IL-18 reduced alveolar thickening and subpleural inflammatory foci elicited by BLM instillation in both wild-type and IL-18−/− mice (Fig. 4B).

Effect of pretreatment with IL-18 on BLM-induced increases in hydroxyproline content in the lung. We used lung hydroxyproline levels as an index of total lung collagen and fibrosis. We found that BLM instillation caused increases in the hydroxyproline content twofold at day 21 and that pretreatment with IL-18 reduced the BLM-induced increase by 30% (Fig. 5).

Effect of pretreatment with IL-18 on BLM-induced increases in cell infiltration and MPO activity in BALF. Effect of pretreatment with IL-18 on the number of infiltrated cells in BALF was analyzed 7 days after BLM instillation in wild-type and IL-18−/− mice. IL-18 pretreatment reduced the total number of infiltrated cells in both types of mice (Fig. 6A). IL-18 pretreatment effectively prevented infiltration of macrophages although infiltration of lymphocytes and neutrophils was not

Fig. 2. BLM-induced increases in cell infiltration (A) and MPO activity (B) in bronchoalveolar lavage fluid (BALF). Total cells, lymphocytes, macrophages, and neutrophils in BALF of wild-type and IL-18−/− mice at 7 and 14 days (D) after instillation with BLM or vehicle were counted. D0 shows total cells before BLM instillation at day 0. Open and closed columns are wild-type and IL-18−/− mice, respectively. Columns and bars express means and SE (n = 3–10). *P < 0.01, **P < 0.01, and ***P < 0.001, comparison between wild-type and IL-18−/− mice by Student’s t-test. MPO activity in BALF at 7 days after instillation of BLM (closed column) or vehicle (open column). Columns and bars express means ± SE (n = 3). **P < 0.01, comparison between vehicle-treated mice and BLM-treated mice in each strain by Student’s t-test; #P < 0.05, comparison between wild-type and IL-18−/− mice by Bonferroni multiple-comparison test.
affected (Fig. 6A). BLM-induced MPO activity in BALF was also significantly reduced by the pretreatment with IL-18 in both types of mice (Fig. 6B).

**Effect of pretreatment with IL-18 on GM-CSF and IL-10 content in the lung.** BLM instillation caused reduction of lung GM-CSF content at day 7, which continued to day 21 (Fig. 7A). IL-18 did not change the lung GM-CSF content (Fig. 7A); however, administration of IL-18 before BLM instillation resulted in prevention of BLM-induced decreases in the lung GM-CSF content (Fig. 7A). Administration of IL-18 did not significantly increase lung IL-10 content (Fig. 7B). BLM instillation caused reduction of IL-10 content at day 7 but not day 21 (Fig. 7B). However, pretreatment of IL-18 reduced BLM-induced reduction of lung IL-10 content at day 7, and at day 21, IL-10 content was higher in BLM-instilled mice than vehicle-treated mice (Fig. 7B).

**Effect of pretreatment with IL-18 on Mn-SOD mRNA expression in the lung.** Superoxide anions have been reported to mediate lung injuries induced by BLM (36). We investigated the effect of IL-18 on the expression of SOD in the lung of wild-type and IL-18/−/− mice. RT-PCR analysis detected weak expression of Mn-SOD mRNA in the lung, and IL-18 treatment increased SOD mRNA levels 1.3-fold in both types of mice (Fig. 8A). BLM treatment decreased SOD activity in the lung of both types of mice (Fig. 8B). IL-18 treatment by itself did not affect SOD activity (data not shown), but treatment with IL-18 before BLM instillation resulted in significant increases in SOD activity compared with BLM-treated mice with no IL-18 pretreatment in both types of mice (Fig. 8B).

### DISCUSSION

BLM induces lung injury through two phases. The first phase involves inflammation of the lung characterized by infiltration of inflammatory cells, such as macrophages, neutrophils, and lymphocytes. This is followed by the second phase, which involves fibrosis characterized by deposition of collagen (15).
We have previously demonstrated that repetitive treatment of mice with IL-18 increases the number of neutrophils in the circulation and suggested that treatment with IL-18 may be useful for reducing a risk of leukopenia in cancer chemotherapy (23). In this study, we found that intratracheal instillation of BLM caused elevation of IL-18 levels in the lung from 33.75\(\pm\)3.07 to 138.33\(\pm\)63.12 pg/mg protein in 24 h, and BLM caused greater lung injury and weight loss and higher mortality in IL-18\(^{-/-}\) mice than in wild-type mice (data not shown). These results suggested that IL-18 may play a protective role in BLM-induced lung injuries. To further examine this possibility, we examined whether administration of IL-18 could ameliorate the lung injuries caused by BLM. In our preliminary experiments, we examined the timing of IL-18 administration. We found that therapeutic administration of IL-18 after BLM instillation had no effect, but prophylactic administration of IL-18 before BLM instillation reduced the mortality, lung hyperplasia, inflammatory cell invasion, and induction of MPO activity. These results strongly suggest that IL-18 suppresses early inflammation induced by BLM.

BLM-induced early inflammation is characterized by infiltration of inflammatory cells in the lung. In the present study, we observed significant increases in the number of macrophages and lymphocytes in the BALF after treatment with BLM. Although we did not detect the significant increase in the number of neutrophils, we could detect the MPO activity, which is thought to show traces of neutrophils in the BALF cells after BLM instillation (2). The number of infiltrated cells reached a peak at day 7 after BLM-instillation in wild-type mice, whereas it continued to increase to day 14 in IL-18\(^{-/-}\) mice. In addition, the increase in the MPO activity was greater...

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**Fig. 5.** Effect of pretreatment with IL-18 on hydroxyproline content in the lung 21 days after BLM instillation. Wild-type mice were treated with IL-18 (2 \(\mu\)g/mouse) 6 and 3 days before BLM instillation and killed at 21 days after BLM instillation. Open column shows vehicle-treated control, closed column BLM-instilled mice, and hatched column IL-18-pretreated and BLM-instilled mice. ### \(P < 0.001\), comparison between BLM-instilled mice and IL-18-pretreated and BLM-instilled mice by Bonferroni multiple comparison.

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**Fig. 6.** Effect of pretreatment with IL-18 on hydroxyproline content in the lung 21 days after BLM instillation. Wild-type mice were treated with IL-18 (2 \(\mu\)g/mouse) 6 and 3 days before BLM instillation and killed at 21 days after BLM instillation. A: infiltrated cells. Open columns show the control group, closed columns the BLM-instilled group, and hatched columns the IL-18-pretreated and BLM-instilled group. * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\), compared with each vehicle control; ### \(P < 0.001\), comparison between the BLM-instilled group and the IL-18-pretreated and BLM-instilled group by Bonferroni multiple comparison test. ND, not detected. B: MPO activity in BALF. Open column shows the control group, closed column the BLM-instilled group, and hatched column the IL-18-pretreated and BLM-instilled group. ** \(P < 0.01\), *** \(P < 0.001\), compared with each vehicle control group; ### \(P < 0.001\), comparison between the BLM-instilled group and the IL-18-pretreated and BLM-instilled group by Bonferroni multiple comparison test.
in IL-18−/− than wild-type mice, suggesting that the degree of early inflammation induced by BLM was greater in IL-18−/− than in wild-type mice. Pretreatment of IL-18 reduced the infiltration of macrophage and neutrophils but not lymphocytes in both wild-type and IL-18−/− mice. The increase in MPO activities was also reduced by IL-18 in both types of mice. These results suggest that IL-18 plays a protective role in BLM-induced lung inflammation and that innate immunity is more crucial than the acquired immunity in BLM-induced lung inflammation. It is not clear why IL-18 reduced the infiltration of macrophages and neutrophils but not lymphocytes, but it is possible that macrophages and neutrophils are more sensitive to ROS generated by BLM than lymphocytes.

Administration of BLM causes destruction of the lung architecture leading to pulmonary fibrosis characterized by increases in hydroxyproline levels and collagen deposition in the lung. Pretreatment of IL-18 reduced hydroxyproline content in the lung, the severity of interstitial pneumoniae, and pulmonary fibrosis. These observations indicated that the administration of IL-18 exerts protective effects against late fibrosis as well as early inflammation in the lung caused by BLM.

It has been reported that several cytokines such as IL-10 and GM-CSF could modulate BLM-induced lung injury. IL-10 inhibits production of proinflammatory cytokines, ROS, and nitric oxide (9). IL-10 may also cause apoptosis of activated neutrophils (8). In the lung injury induced by immune complexes, IL-10 intratracheally instilled has been reported to suppress TNF-α synthesis (30) and silica-induced lung inflammation (14). In IL-10−/− mice, clearance of infected Pneumocystis carinii is facilitated, but the lung damage induced by P. carinii infection is accelerated and enhanced by the absence of IL-10 (29). Introduction of the IL-10 gene has been shown to reduce the severity of lung injury induced by BLM (2). In this study, we found that BLM instillation reduced IL-10 content in the lung and IL-18 pretreatment prevented this reduction, which may contribute to amelioration of the acute lung inflammation.

Administration of GM-CSF has been shown to reduce hydroxyproline in mice with BLM-induced pulmonary fibrosis (27). In GM-CSF−/− mice, BLM caused an enhanced fibrotic response, suggesting that GM-CSF downregulates fibrogenesis
through induction of prostaglandin E2 synthesis (21). These results suggest that the mode of action of GM-CSF in preventing BLM-induced fibrosis may be different from that of IL-10. In the present study, lung GM-CSF content was significantly reduced 7 days after BLM instillation and not recovered until 21 days after BLM instillation. Although IL-18 administration did not elevate lung GM-CSF levels in mice, IL-18 administered before BLM instillation attenuated the reduction of GM-CSF content throughout the 21-day experimental period (Fig. 7A). Alveolar epithelial cells are the source of GM-CSF (7), and therefore, reduction of lung GM-CSF content can be an indicator of alveolar epithelial damage. BLM has been shown to cause severe damage to alveolar epithelial cells (31). Thus it is possible that the suppression of BLM-induced reduction of GM-CSF by IL-18 is the consequence of the protection of alveolar epithelium from BLM-induced injury rather than the induction of GM-CSF.

BLM exhibits its antineoplastic effect through the formation of BLM-iron complexes with ROS, such as superoxide anions (31). ROS induce expression of selectins on endothelia to recruit leukocytes to the lung (1). ROS also stimulate fibroblasts to secrete collagen (5). Uncontrolled generation of ROS by inflammatory cells is considered to cause excessive fibrosis in healing process. The fibrogenic role of ROS in BLM-induced lung injury has been supported by studies showing that administration of SOD, an oxygen free radical-scavenging metalloenzyme, suppresses the progression of lung fibrosis and elevates hydroxyproline levels in interstitial pneumonia caused by BLM in mice (38). These findings suggest that ROS produced by inflammation damage epithelial cells, leading to lung fibrosis. Thus SOD can be a primary defense against ROS to blunt the initial inflammation.

Mn-SOD, which catalyzes the conversion of superoxide radicals to H2O2 in mitochondria (12), is important for tissue protection from oxidative damages during inflammation. In rat models of adjuvant-induced arthritis and BLM-induced lung fibrosis, Mn-SOD, which is stable in blood, has been shown to be more effective as an anti-inflammatory agent than Cu/Zn-SOD with a shorter half-life (26). Consistent with this, Mn-SOD−/− mice cannot survive due to severe lung damages and neurodegeneration (13). It has been reported that IL-1 pretreatment protects mice from BLM-induced pneumonia through induction of Mn-SOD (39). However, no information is available on SOD activity in BLM-instilled mice.

In this study, we found that administration of IL-18 induced Mn-SOD mRNA in the lung. BLM instillation decreased lung SOD activity, but it was reversed by the treatment with IL-18 before BLM. These results indicate that IL-18 can protect the lung from oxidative stresses caused by BLM through upregulation of Mn-SOD. In addition, reduction of lung SOD activity by BLM was much more pronounced in IL-18−/− mice than in wild-type mice, supporting the role of SOD in the protection of lung tissue. Several studies have demonstrated that inflammatory cytokines, including IFN-γ, TNF-α, and IL-1, singly or in combination, induce Mn-SOD (11, 33). Because IL-18 can augment some of these cytokines, IL-18 may upregulate production of Mn-SOD through induction of these cytokines. Further analysis of the mechanism for suppression of BLM-induced lung fibrosis by IL-18 will be beneficial for finding ways to minimize the side effect of BLM in chemotherapy.

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REFERENCES


