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Effects of edaravone, a free radical scavenger, on bleomycin-induced lung injury in mice

Short title: EDARAVONE AND LUNG INJURY

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ABSTRACT

Reactive oxygen species (ROS) play an important role in the pathogenesis of acute lung injury and pulmonary fibrosis. We hypothesized that edaravone, a free radical scavenger, is able to attenuate bleomycin (BLM)-induced lung injury in mice by decreasing oxidative stress.

Lung injury was induced in female ICR mice by intratracheal instillation of 5 mg/kg of BLM. Edaravone (300 mg/kg) was given by intraperitoneal administration at 1 h before BLM challenge.

Edaravone significantly improved the survival rate of mice treated with BLM from 25% to 90% ($p=0.002$), reduced the number of total cells and neutrophils in bronchoalveolar lavage fluid (BALF) on Day 7 ($p<0.05$), and attenuated the concentrations of lipid hydroperoxide (LPO) in BALF and serum on Day 2 ($p<0.05$). The fibrotic change in the lung on Day 28 was ameliorated by edaravone, as evaluated by histologic examination and measurement of hydroxyproline contents ($p<0.05$). In addition, edaravone significantly increased the prostaglandin E_2 (PGE_2) concentration in BALF on Day 2 ($p=0.043$).

In summary, edaravone was shown to inhibit lung injury and fibrosis *via* the repression of LPO production and the elevation of PGE_2 production in this experimental murine system.

KEY WORDS

Bleomycin, edaravone, free radical scavenger, lung injury, pulmonary fibrosis

SHORT TITLE

EDARAVONE AND LUNG INJURY

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic fibrosing interstitial pneumonia limited to the lung [1]. The etiology of IPF is not known, and IPF remains a devastating disease with a more than 50% 5-yr mortality rate [1]. Unfortunately, the pathogenesis of IPF is also incompletely understood. Although several drugs have been used or tried for IPF, there is no established treatment that definitely improves its outcome [1]. Thus new therapies are awaited, based on new understanding of the pathogenesis of IPF. There is considerable evidence that oxygen-generated free radicals play a major role in inflammatory and immune-mediated tissue injury [2-4]. Demedts et al. have shown that acetylecystein, a precursor of the major antioxidant glutathione, given at a daily dose of 1800 mg in combination with prednisone and azathioprine, preserves vital capacity and carbon monoxide diffusing capacity in patients with IPF better than the combination of prednisone and azathioprine alone [5]. These findings suggest that an oxidant-antioxidant imbalance may contribute to the disease process in IPF.

Bleomycin (BLM), an antineoplastic agent, induces pulmonary fibrosis as an adverse effect since the hydrolase that inactivates BLM is relatively scarce in lung tissue. The mechanism of the antineoplastic effect of BLM is that the BLM-iron complex reduces molecular oxygen to superoxide and hydroxy radicals that can then attack DNA and cause strand cleavage [6]. The role of oxygen free radicals has been supported by studies showing that the addition of superoxide dismutase, an oxygen free radical scavenger, inhibited BLM-induced DNA breakage and cellular damage in vitro [7-10]. Therefore, a BLM-induced pulmonary fibrosis model in mice is a helpful tool

to examine the general mechanism of fibrosis, especially that mediated by oxygen free radicals.

Edaravone (3-methyl-1phenyl-2-pyrazoline-5-one) is a potent free radical scavenger and has the antioxidant ability to inhibit lipid peroxidation [11]. It is therefore speculated that edaravone administration might ameliorate the tissue damage induced by reactive oxygen species (ROS). Edaravone has protective effects on both hemispheric embolization and transient cerebral ischemia, and has therefore been used clinically to treat acute brain infarction in Japan [12-14]. Ito et al. have shown that edaravone ameliorated the lung injury induced by intestinal ischemia/reperfusion. In their study, edaravone decreased the neutrophil infiltration, the lipid membrane peroxidation, and the expression of interleukin (IL)-6 mRNA in the lungs, resulting in a reduction in mortality [15]. Most recently, Asai et al. have shown that edaravone suppressed BLM-induced acute pulmonary injury in rabbits [16]. They reported that a 10-day intravenous edaravone administration beginning 3 days prior to intratracheal instillation of BLM significantly attenuated the acute BLM-induced lung injury, and significantly attenuated the numbers of both TUNEL-positive (apoptotic) and transforming growth factor-β-positive cells at Day 7 [16]. Although their results support our hypothesis, we thought several critical points were lacking as follows. 1) They did not evaluate collagen accumulation at the late fibrosing stage. 2) They did not perform bronchoalveolar lavage (BAL) or measure ROS in order to evaluate inhibitory effects on the inflammatory process. Accordingly, in the present study we used a BLM-induced pulmonary fibrosis model in mice, which is a more common animal lung fibrosis model than the rabbit model used by Asai et al., to investigate the

ability of edaravone to 1) inhibit pulmonary fibrosis, 2) decrease lung inflammation, and attenuate ROS.

MATERIALS AND METHODS

Mice, cells, 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/Guide-excerpts.pdf). The study protocol was approved by the Ethics Committee of Jichi Medical University, Tochigi, Japan. Female ICR mice, 6-8 weeks of age, were obtained from Japan SLC (Tochigi, Japan) and housed in the animal facility of Jichi Medical School. BLM was purchased from Nippon Kayaku (Tokyo, Japan). Edaravone was a gift from Mitsubishi Pharma Corporation (Tokyo, Japan). It was dissolved in a small amount of 1N NaOH solution, the pH was adjusted to 7.0 with 1 N HCl, and the concentration was adjusted to 3 mg/ml in the saline solution.

BLM-induced pulmonary fibrosis model

To induce pulmonary fibrosis, we treated ICR mice with intratracheal BLM on Day 0. The ICR mice were anesthetized by the intraperitoneal administration of 0.01 ml/g of 10% pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL, USA), followed by intratracheal instillation of 5 mg of BLM/kg body weight in 50 μl of sterile isotonic saline. The control animals received intratracheal saline only. Edaravone dissolved in saline or the same volume of saline was administered by a single intraperitoneal injection, 1 h before or 24 h after BLM injection. To decide the optimal dose of edaravone for the proposed experiment, mice were given edaravone at a dose of 0, 3, 30, or 300 mg/kg or the same volume of saline (n=10~12 in each group). The

mice were killed under anesthesia on Day 2, 7, or 28 after BLM instillation for examination. On Day 28, the left lung lobes were used for hydroxyproline assay. In the mice receiving pre-administration of 300 mg/kg of edaravone with BLM instillation, bronchoalveolar lavage (BAL) was performed on Days 2 and 7. In addition, histologic examination was performed on Day 28. We selected 6 or 10 mice samples randomly from each group. Mortality study and another experiments (hydroxyproline assay, histologic examination, and BAL analysis) were performed independently.

Sampling of bronchoalveolar lavage fluid and serum

Under anesthetization as previously described, blood samples were obtained from the right atrium at each time point. After centrifugation at 3,000*g* for 10 min at 4^{o}C, the serum was frozen and stored at –80^{o}C until it was assayed. BAL was performed through a tracheal cannula with 0.7 ml saline four times. In each mouse examined, approximately 2.5 ml (90%) of BAL fluid (BALF) was recovered. A 100-μl aliquot was used for the total cell count, and the remainder was immediately centrifuged at 1,000 rpm for 10 min. The total cell count was made using a hemocytometer, and cell differentiation was determined for more than 500 cells on cytocentrifuge slides with Wright-Giemsa staining. The supernatants of BALF were stored at –80^{o}C until used.

Morphologic evaluation

Histopathologic evaluation was performed on Day 28 in the BLM-induced pulmonary fibrosis model. Both lungs were removed and inflated with 10% formaldehyde neutral buffer solution, and longitudinal tissue sections were stained with hematoxylin-eosin.

Assay of hydroxyproline

Hydroxyproline in the murine lung on Day 28 after BLM instillation was assayed according to the commonly used procedure of colorimetric measurement by Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. (Tokyo, Japan) [17, 18]. Hydroxyproline content (μg/lung) was measured in the left lung of each subject.

Assays for lipid hydroperoxide, and prostaglandin E_2

The concentrations of lipid hydroperoxide (LPO) in serum and BALF were measured as an indicator of oxidative stress using a Lipid Hydroperoxide Assay kit (Cayman Chemical, Ann Arbor, MI, USA). Prostaglandin E_2 (PGE_2) in BALF was quantitated using specific immunoassays (Cayman Chemical, Ann Arbor, MI, USA).

Statistical analysis

Survival curves were estimated by the Kaplan-Meier method. Comparisons of all curves were done using the two-tailed log-rank test. Data were expressed as the means ± SEM. For multiple comparisons, we performed a one-way analysis of variance, and then Fisher's protected least-significant differences method was used as a post-hoc test. Differences between two variables were assessed with the Mann-Whitney U-test. Values of $P<0.05$ were considered to indicate statistical significance.

RESULTS

Edaravone caused a significant reduction in the mortality of mice with BLM-induced pulmonary fibrosis

The severe lung injury caused by BLM administration was associated with high mortality. To assess the protective effects of edaravone, the compound was injected intraperitoneally in various doses at various times either before or after the BLM instillation. The survival rate of each group is shown in fig. 1. Nine of 12 animals (75%) died from Day 3 to 20 after treatment with 5 mg/kg of BLM. The pre-administration of 300 mg/kg of edaravone, however, significantly improved the survival rate of mice treated with BLM (one of 10 animals died, $p=0.002$) (fig. 1). In contrast, among the mice treated with low dose-edaravone (pre-administration of 3 or 30 mg/kg) followed by BLM instillation, only 3 of 10 survived in both dosage groups (fig. 1). The administration of 300 mg/kg of edaravone after 24 h BLM injection (post-treatment administration = treatment group) did not improve the survival rate of mice treated with BLM (5 of 11 animals died, $p=0.15$) (fig. 1).

Administration of edaravone ameliorated BLM-induced pulmonary fibrosis in mice

To evaluate the antifibrotic effect of edaravone, mice were treated with 5 mg/kg of BLM and killed on Day 28. The fibrotic change in the lung was evaluated by histologic examination and measurement of hydroxyproline contents. As shown in Fig. 2, when 300 mg/kg of edaravone was administered before BLM-instillation, a significant reduction of fibrosis in the subpleural areas of the lung was observed. The hydroxyproline assay demonstrated that the pre-treatment with edaravone

dose-dependently reduced the total hydroxyproline contents in BLM-treated lungs (fig. 3). The post-treatment administration (treatment group) of 300 mg/kg of edaravone was also effective in reducing the pulmonary fibrosis caused by BLM.

Analysis of BALF cells in mice with BLM-induced pulmonary fibrosis

Next, we analyzed the cells in BALF to evaluate the effects of edaravone on the inflammatory responses induced by BLM. Edaravone (300 mg/kg body weight) was administered by a single intraperitoneal injection 1 h before BLM injection. Administration of BLM elevated the number of inflammatory cells, including macrophages, lymphocytes, and neutrophils, on Days 2 and 7. Pre-administration of edaravone significantly reduced the number of total cells and neutrophils in BALF on Day 7 ($p<0.05$) (fig. 4a and c). As shown in fig. 4a and c, the *p* value of total cells and neutrophils in BALF between BLM group and BLM+edaravone group were significant but marginal ($p=0.045$ and $p=0.046$, respectively). Therefore, we did not perform BALF cell analysis, measurement of LPO or PGE2, without pretreatment of 300 mg/kg of edaravone.

Effects of edaravone on the amount of lipid hydroperoxide in serum and BALF in the BLM model

One of the possible reasons for the preventive effect of edaravone on BLM-induced lung injury may be its antioxidant effect. To study the antioxidant effect of edaravone, we measured the amount of LPO in the serum and BALF, which is an indicator of oxidative stress [19]. On Day 2 after BLM instillation, serum LPO levels were significantly increased compared to those in the control mice ($p=0.013$) (fig. 5a). On

the other hand, pre-treatment with edaravone (300 mg/kg body weight) significantly decreased the levels of LPO in serum, compared with those in the animals treated with BLM alone ($p=0.001$) (fig. 5a). LPO production in BALF was also significantly lowered by edaravone injection on Day 2 ($p=0.049$) (fig. 5b). The serum or BALF levels of LPO in edaravone-treated mice on Day 7 after BLM challenge did not differ from those in untreated mice (data not shown).

Effects of edaravone on the PGE_2 levels in BALF of the BLM model

We measured the PGE_2 level in BALF as an index of the amount of anti-inflammatory prostanoids. PGE_2 was measured by immunoassay in BLM-treated mice with or without pre-treatment of edaravone (300 mg/kg body weight). As shown in fig. 6, mice pre-treated with edaravone exhibited significantly greater levels of PGE_2 than did mice receiving BLM alone on Day 2, but this elevation of PGE_2 by edaravone thereafter rapidly decreased until Day 7 (data not shown).

DISCUSSION

We have shown that the anti-inflammatory effects of edaravone improved the 28-day survival in mice with acute lung injury after a BLM instillation. Edaravone could mitigate the progression of lung injury and fibrosis. It also attenuated the cellular infiltration and the concentrations of LPO in BALF. These findings suggested that edaravone could inhibit lung injury and fibrosis *via* the repression of LPO production in our model.

In this study we used a murine BLM-induced pulmonary fibrosis mouse model and examined the ability of edaravone to 1) inhibit pulmonary fibrosis, 2) decrease lung inflammation, and attenuate ROS. First, we investigated the ability of edaravone to inhibit pulmonary fibrosis using histologic examination and measurement of hydroxyproline contents. We found that a single administration of edaravone not only when 1 h before, but also when 24h after BLM challenge could mitigate the progression of pulmonary fibrosis on Day 28 after BLM instillation. Second, we investigated the ability of edaravone to decrease lung inflammation, and attenuate ROS. Our present study demonstrated that edaravone could attenuate the concentrations of LPO (an indicator of oxidative stress) in BALF and serum on Day 2. An oxidant-antioxidant imbalance may contribute to the pathogenesis of BLM-induced pulmonary fibrosis [7-10]. Hagiwara et al. have shown that aerosolized administration of N-acetylcysteine (NAC) attenuates lung fibrosis induced by BLM *via* repression of LPO production [9]. In the present study, the number of total cells and neutrophils in BALF in edaravone-treated mice on Day 7 was significantly decreased in comparison to that in untreated mice. These findings were consistent with the previous reports [8-10]. Most

of the antioxidant agents used for treatment of BLM models have shown not only anti-fibrosing effects but also anti-inflammatory effects—i.e., attenuating the cellular infiltration, proinflammatoy cytokines or chemokines in BALF [8-10]. Although we did not measure proinflammatoy cytokines or chemokines in BALF, we speculate that edaravone may have decreased the proinflammatory cytokine or chemokine production in our BLM-induced lung injury model.

Our present study demonstrated that a single administration of edaravone reduced the total hydroxyproline contents in BLM-treated lungs on day 28. Although numerous agents targeting diverse signaling and molecular pathways inhibited fibrosis very effectively in BLM-induced pulmonary fibrosis model, none of these molecules has thus far demonstrated clear efficacy in the treatment of IPF. A main difference between the human disease and the mouse model is the inflammatory component of early BLM-induced lung injury, which is often absent in human IPF [20]. Recently, Chaudhary et al. determined the time course of the development of inflammation and fibrosis in BLM-induced lung fibrosis [21]. They demonstrated that in an animal model of single intratracheal injection of BLM, the “switch” between inflammation and fibrosis occurred on or just after Day 9 [21]. Although we tried daily intravenous or intraperitoneal injections of 60 mg/kg of edaravone from 14 days after BLM instillation, there was no beneficial effect (data not shown). Hagiwara et al. used NAC inhalation and obtained results similar to those in our study [9]. Our results suggested that edaravone might not show a therapeutic effect on chronic fibrotic lung diseases such as IPF, but may have a preventive effect in the very accelerated phases of interstitial lung diseases, such as in acute exacerbation of IPF, acute interstitial pneumonia, or drug-induced lung diseases.

Watanabe et al. have shown that edaravone acts as: 1) a radical scavenger, 2) a stimulator of prostaglandin production, 3) an inhibitor of lipoxygenease and 4) a protector against cell membrane damage [22]. We thus considered that arachidonic acid might be preferentially metabolized *via* the alternative cyclooxygenase (COX) pathway to prostanoids that possess anti-inflammatory and anti-fibrotic activity; the best characterized of these prostanoids is PGE_2. PGE_2 is produced in large quantities by macrophages in response to proinflammatory molecules such as IL-1 and LPS [23-25] and is therefore also considered a proinflammatory mediator. In addition to its effects on inflammation, PGE_2 suppresses fibroblast proliferation [26] and reduces collagen mRNA expression [27], thereby exerting an antifibrotic activity. In vivo, consistent with an anti-fibrotic activity of PGE_2, COX2 knockout mice were found to be more susceptible to BLM-induced lung fibrosis [28]. We found that the administration of edaravone before BLM challenge produced more PGE_2 in the BALF than saline administration. Egan et al. have shown that the COX-PG pathway is irreversibly self-deactivated due to the natural reduction of the hydroperoxide at carbon 15 of PGG_2 to the hydroxyl on PGH_2 [29]. During this reduction, radicals, possibly hydroxyl radicals, are formed and could oxidize the enzyme [29]. Therefore, edaravone may increase both the initial rate and the total reaction prior to deactivation by partially consuming these radicals. We did not examine which cells (macrophages, epithelial cells, endothelial cells, or fibroblasts) contribute to PGE_2 production. Further examination will be needed to determine which cells were affected by edaravone.

Usually, the daily dose of edaravone in humans is approximately 1.5 mg/kg, and the duration of medication is 14 days after cerebral infarction [11-14]. Although, in a previous report, no adverse effects on heart rate or blood pressure at the dose of 450

mg/kg of edaravone were reported [30], we observed a temporary increase of serum creatinine levels at the dose of 300 mg/kg of edaravone (fig. 7). However, the creatinine elevation at Day 2 after BLM instillation was normalized until Day 7 (fig. 7). We did not observe other adverse effects of a single administration of 300 mg/kg/day of edaravone in this study, despite the fact that this dose was roughly 200 times the daily dose used in humans. Anzai et al. have shown that a radioprotective effect of edaravone against whole body X-ray irradiation in C3H mice [30]. To increase the survival rate, the necessary dose of edaravone was 450 mg/kg intraperitoneally, and the timing of the administration was 30 min prior to the irradiation [30]. Asai et al. used daily intravenous injections of 3 mg/kg of edaravone for rabbits administered 2 mg/kg bleomycin [16]. In the present study, we required a high dose of edaravone for the treatment of lung injury in ICR mice. In addition to the dose-dependency, the efficacy of edaravone in ameliorating BLM-induced organ injury was also dependent on the administration route and the strain of mice.

In summary, the results of the present study suggested that edaravone could inhibit BLM-induced lung injury and fibrosis *via* the repression of LPO production and augmentation of PGE_2 production. Additional clinical studies on other fatal interstitial lung diseases such as acute exacerbation of IPF, acute interstitial pneumonia associated with collagen vascular diseases, or chemotherapy related toxicity will be needed to determine the safest dose, administration route, and duration times of edaravone.

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FIGURE LEGENDS

FIGURE 1. Effects of edaravone on mortality in a BLM-induced lung injury mouse model. The survival rates of five study groups of mice are shown over a 28-day observation period (n=10~12 in each group). The group given intratracheal instillation of BLM is shown by *closed circles*; the four BLM+Edaravone groups received single intraperitoneal infusion of edaravone as follows. A high dose of edaravone (pretreatment, 300 mg/kg; *open circles*), intermediate dose of edaravone (pretreatment, 30 mg/kg; *open triangles*), or low dose of edaravone (pretreatment, 3 mg/kg; *closed triangles*) was given as a single intraperitoneal infusion at 1 h before the instillation of BLM. The fourth experimental group received a high dose of edaravone as a single intraperitoneal infusion at 24 h after the instillation of BLM (treatment, 300 mg/kg; *crosses*). The survival rate of the high-dose edaravone (pretreatment, 300 mg/kg; *open circles*) group was significantly higher than that of the BLM (*closed circles*) group ($p < 0.05$). The results for the control group are not shown.

Fig. 1. Tajima S. Top ↑

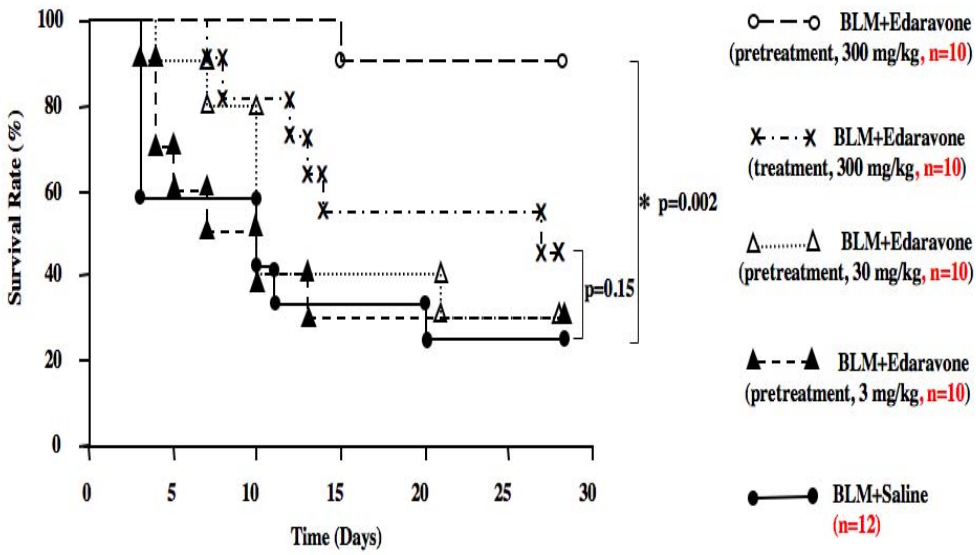

FIGURE 2. Effects of edaravone on histopathologic changes. Lung tissue was obtained on Day 28 after instillation of BLM or saline and was stained with H & E [original magnification ×100]. (a) A lung tissue sample from the saline group shows thin interalveolar septa, a lack of inflamed cells, and normal-appearing bronchioles and alveolar ducts. (b) A lung tissue sample from the BLM group shows alveolitis and patchy fibrosis with destruction of the alveolar structure, mainly in the subpleural regions. (c) However, these features were less severe in the mice pretreated with a high dose (300 mg/kg) of edaravone.

Fig. 2. Tajima S. Top ↑

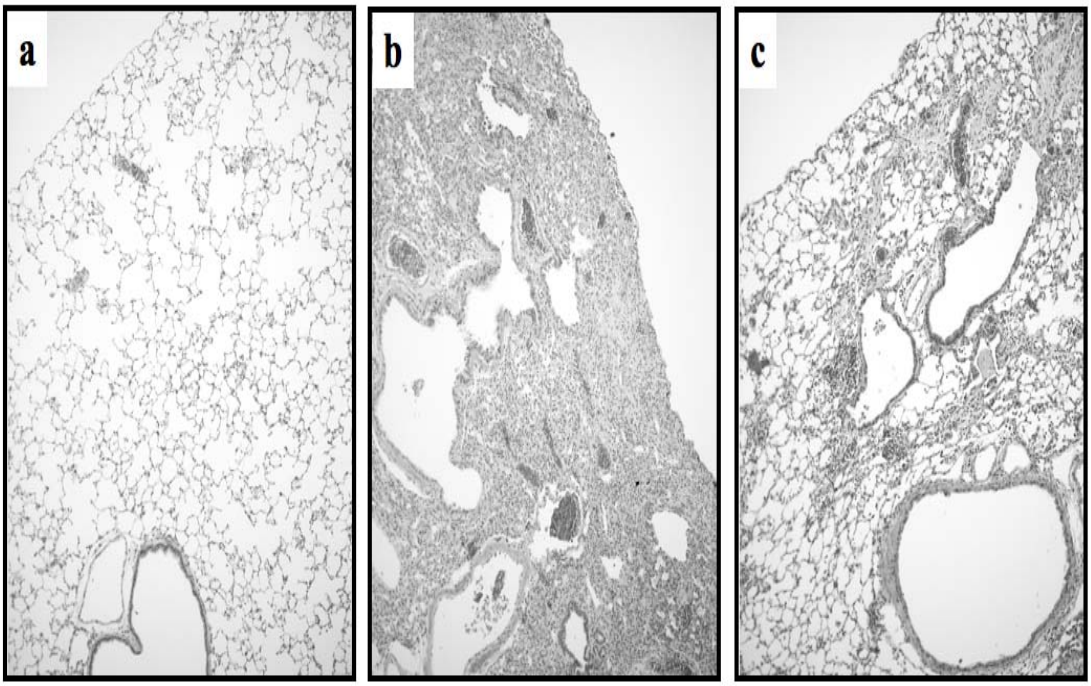

FIGURE 3. Effects of edaravone (white bar: Control group; gray bar: BLM group; black bar: BLM+Edaravone group) on the hydroxyproline content in the left lung in a BLM-induced pulmonary fibrosis mouse model. The hydroxyproline content was significantly increased by BLM injection. Single administration of 30 or 300 mg/kg of edaravone 1 h before BLM instillation significantly attenuated the BLM-induced increase in hydroxyproline content on Day 28. In addition, a single high dose (300 mg/kg) of edaravone by intraperitoneal infusion at 24 h after the instillation of BLM also significantly decreased hydroxyproline contents. Data are presented as the mean ± SEM (n=6~10 in each group). *$p < 0.05$ in comparison to the BLM group.

Fig. 3. Tajima S. Top ↑

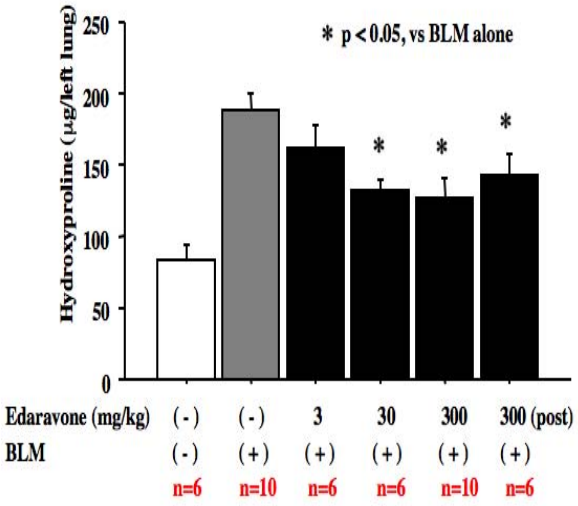

FIGURE 4. Effects of edaravone (white bar: Control group; gray bar: BLM group; black bar: BLM+Edaravone group) on BALF cell analysis in a BLM-induced pulmonary fibrosis mouse model. Single administration of 300 mg/kg of edaravone 1 h before BLM instillation significantly reduced the number of total cells and neutrophils in BALF on Day 7 ($p<0.05$) (a and c). There was no change in the number of macrophages or lymphocytes in BALF on Day 7 (b and d). Data are presented as the mean ± SEM (n=6 in Control and each Day 2 group, n=10 in each Day 7 group). *$p <$ 0.05 in comparison to the BLM group.

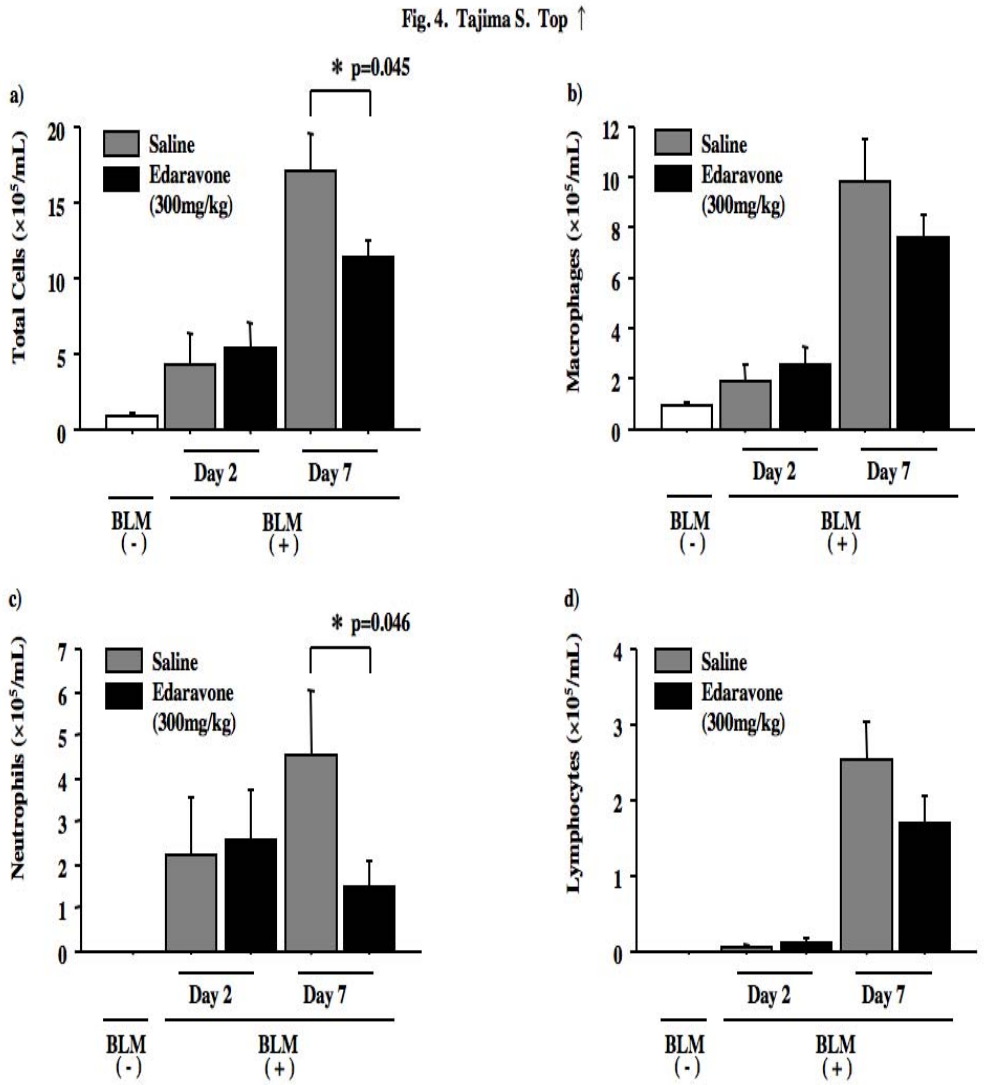

FIGURE 5. Effects of edaravone (white bar: Control group; gray bar: BLM group; black bar: BLM+Edaravone group) on the amount of LPO in serum and BALF in a BLM-induced pulmonary fibrosis mouse model. Edaravone treatment consisted of a single administration of 300 mg/kg at 1 h before BLM instillation. (a) Although on Day 2 after BLM instillation, serum LPO levels were significantly increased compared to the control mice, administration of edaravone significantly decreased the levels of LPO in serum. (b) LPO production in BALF was also significantly lowered by edaravone injection on Day 2. Data are presented as the mean ± SEM (n=6 in each group). *$p < 0.05$ in comparison to the BLM group.

Fig. 5. Tajima S. Top ↑

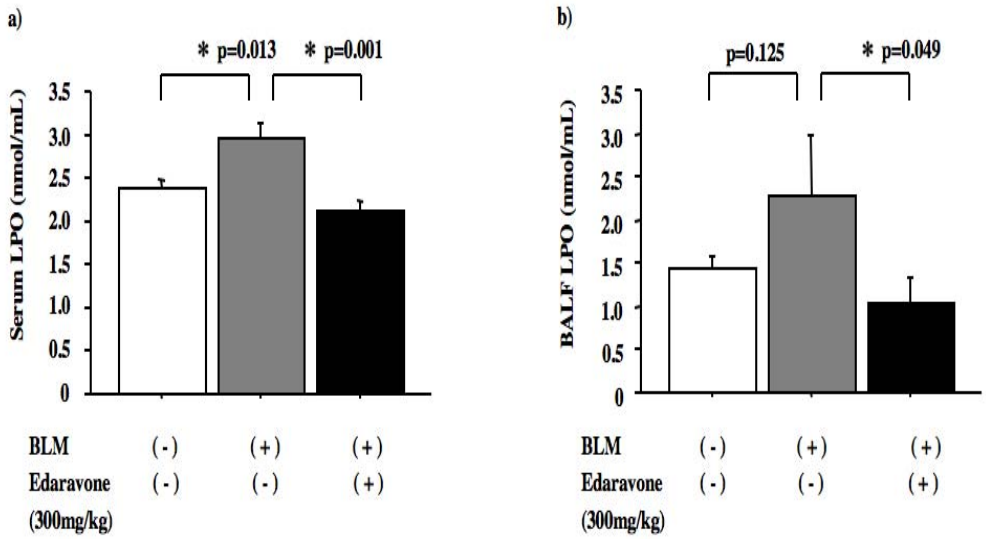

FIGURE 6. Effects of edaravone (white bar: Control group; gray bar: BLM group; black bar: BLM+Edaravone group) on the PGE_2 levels in BALF of a BLM-induced pulmonary fibrosis mouse model. Single administration of 300 mg/kg of edaravone 1 h before BLM instillation significantly increased PGE_2 on Day 2. Data are presented as the mean ± SEM (n=6 in each group). *$p < 0.05$ in comparison to the BLM group.

Fig. 6. Tajima S. Top ↑

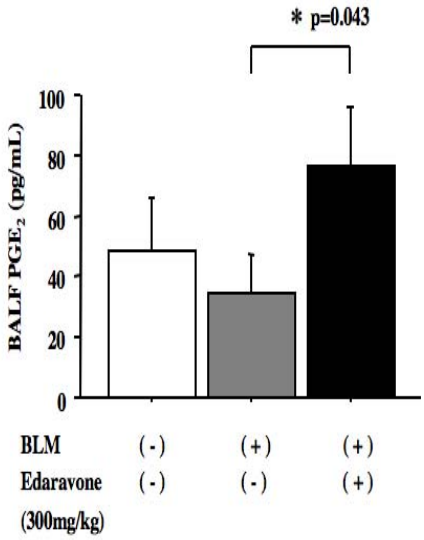

FIGURE 7. Adverse effects of edaravone (white bar: Control group; gray bar: BLM group; black bar: BLM+Edaravone group) on the serum creatinine levels in a BLM-induced pulmonary fibrosis mouse model. The serum creatinine levels were measured by Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. (Tokyo, Japan). Although a temporary increase of serum creatinine levels at the dose of 300 mg/kg of edaravone was observed at Day 2 after BLM instillation, the elevation was normalized until Day 7. Data are presented as the mean ± SEM (n=6 in each group). *$p < 0.05$ in comparison to the Control group.

Fig. 7. Tajima S. Top ↑