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Long-acting human serum albumin-thioredoxin fusion protein suppresses bleomycin-induced pulmonary fibrosis progression

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Running title: HSA-Trx suppresses BLM-induced lung fibrosis

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Abbreviations: IPF, Idiopathic pulmonary fibrosis; HSA, human serum albumin; Trx; thioredoxin 1; HSA-Trx, HSA-Trx fusion protein; BLM, bleomycin; ROS, reactive oxygen species; BALF, bronchoalveolar lavage fluid.

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is thought to involve inflammatory cells and reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$). There is currently no effective treatment for IPF. We previously developed a human serum albumin (HSA)-thioredoxin 1 (Trx) fusion protein (HSA-Trx), designed to overcome the unfavorable pharmacokinetic and short pharmacological properties of Trx, an anti-oxidative and anti-inflammatory protein. In this study, we examined the therapeutic effect of HSA-Trx on an IPF animal model of bleomycin (BLM)-induced pulmonary fibrosis. A pharmacokinetic study of HSA-Trx or Trx in BLM mice showed that the plasma retention and lung distribution of Trx was markedly improved by fusion with HSA. A weekly intravenous administration of HSA-Trx, but not Trx, ameliorated BLM-induced fibrosis as evidenced by a histopathological analysis and pulmonary hydroxyproline levels. HSA-Trx suppressed active-TGF-β levels in the lung and inhibited the increase of inflammatory cells in BALF, pulmonary inflammatory cytokines and oxidative stress markers. An *in vitro* EPR experiment using PMA-stimulated neutrophils confirmed the $O_2^{\cdot-}$ scavenging ability of HSA-Trx. Furthermore, post-treatment of HSA-Trx had a suppressive effect against BLM-induced fibrosis. These results suggest that HSA-Trx has potential as a novel therapeutic agent for IPF, due to its long-acting anti-oxidative and anti-inflammatory modulation effects.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic fibrosing interstitial pneumonia with no identifiable cause. It remains a devastating disease with a 5-year mortality rate of 50% due to its insufficient response to medical therapy. Unfortunately, with the present lack of a complete understanding of the pathogenesis of IPF, the current treatment, which involves the use of steroids and immunosuppressants, does not improve the prognosis and recover from the acute exacerbation of the disease (American Thoracic Society and the European Respiratory Society, 2000; Luppi et al., 2004). Therefore, the development of new drugs designed to suppress the progression of the disease or to prevent the acute exacerbation of IPF is of great importance.

Recent studies have suggested that oxidative stress plays an important role in the pathogenesis and development of IPF. In fact, increased level of reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\cdot-}$) and a decrease in the levels of glutathione and superoxide dismutase (SOD) in blood and in bronchoalveolar lavage fluid (BALF) of IPF patients have been reported (Beeh et al., 2002; Psathakis et al., 2006). In addition, the genetic knockout of NADPH-oxidase, which increases the pulmonary level of $O_2^{\cdot-}$, resulted in the suppression of bleomycin (BLM)-induced pulmonary fibrosis in an IPF animal model. An intratracheal injection of BLM into the lungs of rodents causes alveolar cell damage, an inflammatory response, fibroblast proliferation and subsequent collagen deposition, resembling human fibrotic lung disease (Moore and Hogaboam, 2008). In contrast, the genetic knockout of extracellular SOD, which decreases pulmonary ROS levels, resulted in the progression of the BLM-induced pulmonary fibrosis (Fattman et al., 2003; Manoury et al., 2005). On the other hand, the role of chronic inflammation in the pathogenesis of IPF has been the focus of a number of studies, in view of the presence of interstitial and alveolar inflammatory cells as well as the expression of inflammatory cytokines in the lungs of patients with IPF (Keane and Strieter, 2002). The findings of a large prospective study examining the histopathologic variability of surgical lung biopsies in 109 patients with IPF suggest an evolving disease process with chronic inflammation playing a pathogenic role (Flaherty et al., 2001). In

addition, it has been well established that the levels of inflammatory cytokines and chemokines, especially IL-6, TNF-α and MIF are markedly increased in the lungs of BLM-induced disease model animals, and it has also been reported that the knock out of IL-6 or the administration of anti-TNF-α or MIF antibodies suppress BLM-induced lung disorders (Chaudhary et al., 2006; Piguet et al., 1993; Saito et al., 2007; Tanino et al., 2002). Therefore, drugs with both anti-oxidative and anti-inflammatory properties would be expected to be useful in the treatment of IPF.

Thioredoxin-1 (Trx) is a small redox-active protein (approx. MW of 12 kDa) that is ubiquitously present in the human body and is one of the defense proteins induced in response to various oxidative stress conditions (Holmgren, 1989; Nakamura et al., 2005). In addition to its potent anti-oxidative effect, which is derived from dithiol-disulfide exchange in its active site, Trx also has anti-inflammatory properties, due mainly to its ability to inhibit neutrophil chemotaxis to inflammatory sites and to suppress the expression and activation of the macrophage migration inhibitory factor (MIF) (Nakamura et al., 2001; Tamaki et al., 2006). Because of its desirable anti-oxidative and anti-inflammatory properties, Trx represents a new and potentially effective therapeutic agent for the treatment of IPF. However, since Trx is eliminated extensively via glomerular filtration, its plasma half-life is only about 1 hour in mice and 2 hour in rats, which is extremely short in terms of producing a significant therapeutic impact (Nakamura et al., 2001; Ueda et al., 2006). In order to obtain a satisfactory therapeutic outcome, a sustainable therapeutic concentration of Trx would be needed. To achieve this, constant infusion or frequent repeated administrations of Trx would be required (Hoshino et al., 2003; Liu et al., 2004; Ueda et al., 2006). Hoshino et al. demonstrated that exogenous recombinant Trx was effective in inhibiting BLM-induced lung damage (Hoshino et al., 2003), when administered intraperitoneally, or via a continuous infusion of Trx that is repeated at 2 day intervals.

In an attempt to increase the blood retention time of Trx, we recently produced a genetically engineered fusion protein of human serum albumin (HSA) and Trx (HSA-Trx) using a *Pichia* expression system. The plasma half-life of the HSA-Trx fusion protein in normal mice was found to

be similar with that of HSA, which is 10 times longer than the plasma half-life of Trx (Ikuta et al., 2010). Interestingly, HSA-Trx showed a higher distribution to the lungs than Trx. Therefore, a further attempt will be made to investigate the clinical usefulness of HSA-Trx in treating oxidative stress and inflammation related lung disorders.

The purpose of this study was to investigate the therapeutic impact of HSA-Trx in the treatment of IPF. Using the BLM-induced pulmonary fibrosis animal model, the results showed that HSA-Trx could prevent BLM-induced pulmonary fibrosis via its long acting anti-oxidative and anti-inflammatory modulation effects.

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MATERIALS & METHODS

Materials

BLM was purchased from Nippon Kayaku (Tokyo, Japan). Mayer's hematoxylin, a 1% eosin alcohol solution, mounting medium for histological examinations (malinol), and Masson's trichrome staining reagents were from Muto Pure Chemicals (Tokyo, Japan). Optimal cutting temperature (OCT) compound was purchased from Sakura Finetek (Tokyo, Japan). Chloral hydrate, chloramine T and phorbol myristate acetate (PMA) were obtained from Sigma (Tokyo, Japan). Paraformaldehyde, trichloroacetic acid (TCA), perchloric acid and 4-(dimethylamino)-benzaldehyde (DMBA) was from Nacalai Tesque (Kyoto, Japan). Diethylene-triamine-pentaacetic acid (DTPA) was purchased from Dojindo Laboratories (Kumamoto, Japan). 5, 5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Alexis Biochemicals (Lausen, Switzerland). The TGF-β1 ELISA kit was purchased from R&D Systems Inc (Minneapolis, USA). IL-6 and TNF-α ELISA kit were purchased from Biolegend (San Diego, USA). Sea-ICR mice (6 weeks, male) were obtained from Kyudo Co.,Ltd (Saga, Japan). Other chemicals used were obtained from commercial suppliers.

Production of HSA-Trx fusion protein

Trx and HSA fusion protein was produced following the method reported previously (Ikuta et al., 2010; Furukawa et al., 2011) with a slight modification. The transformed *Pichia pastoris* was incubated in 1.25 L of growth phase media, BMGY (1% yeast extract, 2% pepton, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% glycerol) for 2 day (OD_{600}=2). It was then cultured in 800 mL of protein induction phase media, BMMY (1% yeast extract, 2% pepton, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% methanol) for 3 day at 30ºC. Methanol was added every 24 hour so that the concentration of methanol was maintained at 1% in order to sustain the protein expression induction effect. The

secreted fusion protein was isolated from the growth media as follow. The solution was loaded on to a column of Blue Sepharose 6 Fast Flow column (GE Healthcare, Tokyo, Japan) equilibrated with 200 mM sodium acetate buffer (pH 5.5) after the media was dialyzed against the same buffer. The column was washed with approx. 5 bed volumes of 200 mM sodium acetate buffer, pH 5.5, and then the fusion protein was eluted with 20 mM sodium acetate buffer, pH 6.5, containing 3M NaCl. Next, using AKTA prime, the eluate was loaded on to a column of 5 mL HiTrap Phenyl HP column (GE Healthcare, Tokyo, Japan) for hydrophobic chromatography with the following conditions (Buffer A, 0 mM Tris-HCl/1.5 M ammonium sulfate (pH 7.0); Buffer B, 50 mM Tris-HCl (pH 7.0); Gradient, 0-100% (Buffer B) 100 mL; Flow rate, 3 mL/min). The desired fusion protein was obtained by delipidation with activated carbon treatment, as described by Chen (Chen, 1967). The fusion protein was analyzed by SDS-PAGE and native SDS-PAGE using a 15% polyacrylamide gel, with Coomassie blue R250 staining. The purity of the fusion protein was estimated to be more than 95% (Ikuta et al., 2010).

Production of BLM-induced pulmonary fibrosis mice model

All animal experiments were conducted in accordance with the guidelines of Kumamoto University for the care and use of laboratory animals. BLM-induced pulmonary fibrosis model mice were produced by intratracheal injection of BLM (5 mg/kg) in PBS (1 ml/kg) under anesthesia with chloral hydrate (500 mg/kg) on day 0 (Tanaka et al., 2010). Either Trx or HSA-Trx was administered (but not both) intravenously (3.5 nmol protein in 200 μL PBS/mice) via the mice tail vein 30 minute before BLM treatment (day 0). HSA-Trx was administrated intravenously at day 0 and day 7 (refer to “every 1 week”, Fig. 1a), or only day 0 (refer to “every 2 weeks”, Fig. 1a). For an intervention study, the first dose of HSA-Trx was administered 1 day after the BLM treatment, and the subsequent dose administered at day 7 after the BLM treatment. (Fig. 1c)

Histopathological analysis of lung tissue (HE staining and Masson’s trichrome staining of

collagen)

On day 14 after BLM administration, the whole lungs were flushed with 4% paraformaldehyde before being removed. The removed lungs were fixed in 4% buffered paraformaldehyde and then embedded in paraffin before being cut into 4-μm-thick sections. For HE staining, sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. For Masson's trichrome staining, sections were sequentially treated with solution A [5% (wt/vol) potassium dichromate and 5% (wt/vol) TCA], Weigert's iron hematoxylin, solution B [1.25% (wt/vol) phosphotungstic acid and 1.25% (wt/vol) phosophomolybdic acid], 0.75% (wt/vol) Orange G solution, solution C [0.12% (wt/vol) xylidine ponceau, 0.04% (wt/vol) acid fuchsin, and 0.02% (wt/vol) azophloxin], 2.5% (wt/vol) phosphotungstic acid, and finally an aniline blue solution. HE and Masson's trichrome staining samples were mounted with malinol and inspected with the aid of a microscope (Keyence, BZ-8000, Osaka, Japan).

Determination of lung fibrosis

Fibrosis score was evaluated (×100) as the quantity of the section positively stained for collagen and displaying alveolar wall thickening (1 = <25%, 2 = 25–50%, 3 = 50–75%, and 4 = 75–100%) (Gibbons et al., 2011). Only fields where most of the field was composed of alveoli were scored. The entire lung section was analyzed. The investigator was masked to each sample.

Determination of hydroxyproline level in lung tissues

Hydroxyproline content was determined as described previously (Woessner, 1961). On day 14 after BLM administration, the right lung was removed and homogenized in 1 ml of 5% TCA. After centrifugation, the pellets were hydrolyzed in 0.5 ml of 10 N HCl for 16 hour at 110ºC. Each sample was incubated for 20 minute at room temperature after the addition of 0.5 ml of 1.4% (wt/vol) chloramine T solution and then incubated at 65ºC for 10 minute after addition of 0.5 ml of Ehrlich's reagent [1 M DMBA, 70% (vol/vol) isopropanol and 30% (vol/vol) perchloric acid]. The absorbance was measured at 550 nm to determine the amount of hydroxyproline.

Quantification of activated TGF-β1, IL-6 and TNF-α, and western blot analysis of

macrophage migration inhibitory factor (MIF) in lung tissue

On days 3 and 7 after BLM administration, the whole lungs were removed and homogenized in 0.5 ml of buffer (PBS, 1% protease inhibitor cocktail, 10 mM EDTA, 0.05% Tween-20). After centrifugation at 21,000 g for 10 minute at 4ºC (two times), the supernatants were recovered. The amount of active TGF-β1 (Day 7), IL-6 and TNF-α (Day 3 and Day 7) in the supernatant was measured by ELISA, following the manufacturer's protocol, respectively. In addition, western blotting of MIF chemokine in the supernatant was performed by the following protocol. After measurement of the protein content using the BCA protein assay reagent (Pierce Biotechnology Inc, Rockford, USA), each sample separated by 12.5% SDS-PAGE and transferred onto PVDF membranes (Immobilon-P; Millipore, Bedford, USA) by wet electroblotting. The membranes were blocked for 1 hour at room temperature with 5% skim milk in PBS. The membranes were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and incubated for 2 hour at room temperature with a 400 ng/ml primary mouse monoclonal antibody against N-terminus of MIF of human origin, which has cross reactivity with mouse MIF (Santa Cruz Biotechnology Inc, cat#: sc-271631, Santa Cruz, USA) in PBS-T. The membranes were washed 3 times with PBS-T and incubated with the secondary antibody (horseradish peroxidase-linked anti-mouse IgG (H+L) (Invitrogen, Eugene, USA)) for 1.5 hour at room temperature. The membranes were washed 3 times with PBS-T and immunoblots were visualized using the SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology Inc, Rockford, USA) with LAS-4000EPUVmini (Fujifilm, Tokyo, Japan).

Counting of cells in BALF

On days 1 and 3 after BLM administration, the mice were anesthetized with pentobarbital, the chests were opened and blood was drained. BALF was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 U/ml heparin (two times). About 1.8 ml of BALF was routinely recovered from each animal. The BALF was centrifuged at 4,100 g for 5

minute at 4ºC to separate the cells in the BALF from the liquid. Cells were dissolved in 0.9% NaCl and lysate was centrifuged again. From the recovered cells, the total cell number was counted using a hemocytometer. Cells were stained with Diff-Quick reagents (Kokusai Shiyaku, Kobe, Japan), and the ratios of alveolar macrophages, neutrophils, and lymphocytes to total cells were determined. More than 200 cells were counted for each sample.

Immunostaining of lungs tissue

On day 3 after BLM administration, the whole lungs were flushed with sterile PBS before being removed. The removed lungs were stored in 4% paraformaldehyde at 4ºC for 2 hour before being immersed in a 10% sucrose solution overnight. The concentration of sucrose solution was then adjusted to 20% at room temperature and incubation was continued for another 6 hour. The recovered lungs were covered with OCT compound and frozen at −80ºC. Next, the lungs frozen with cryostat (Leica, CM3000II) were sliced at a thickness of 4 μm and attached on a glass slide. The slide was then cleansed to remove OCT compound and dried. After drying the slide completely, a solution containing 50 mM Tris/HCl + 0.1 % Tween-20 (T-TB) was used to solubilise the lung slice, followed by blocking with Block Ace (Dainippon Pharmaceutics, Osaka, Japan) at room temperature for 15 minute. Next, the primary antibody reaction was conducted below 4ºC overnight. In addition, the primary mouse monoclonal antibody against 8-hydroxy-2'-deoxygenase (8-OH-dG) [15A3] (Santa Cruz Biotechnology Inc, cat#: sc-66036, Santa Cruz, USA) , which has cross-reactivity with mouse or the primary rabbit polyclonal antibody against nitrotyrosine (NO_2-Tyr) (Millipore, cat#: AB5411, Billerica, USA), which has cross-reactivity with mouse was diluted to 2 μg/ml or 20 μg/ml before use, respectively. The lung slices were then washed with 50 mM Tris/HCl (TB) and T-TB, followed by the secondary antibody reaction at room temperature for 1.5 hour. For the secondary antibody, in relation to NO_2-Tyr and 8-OH-dG, Alexa Fluor 546 goat anti-mouse IgG (H+L) (Invitrogen, Eugene, USA) and Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen, Eugene, USA) diluted 200 times were respectively used. After the reaction, the slide

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was observed using a Microscope (Keyence, BZ-8000, Osaka, Japan). Image analyses of the extent and intensity of 8-OH-dG and NO_2-Tyr staining were also performed using imageJ software.

Determination of malondialdehyde (MDA) in lung tissue

On day 3 after BLM administration, the right lung was removed and homogenized in 0.5 ml of RIPA/PI buffer (150 mM NaCl, 1% nonidet-P40, 10 mM Tris-HCl pH7.4 including a 1% solution of a protease inhibitor (Nacalai tesque, Tokyo, Japan)). After centrifugation, the amount of MDA in supernatant was measured by thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical, Ann Arbor, USA), which is well-established method for measuring lipid peroxidation, according to the manufacturer's protocol.

Isolation of polymorphonuclear neutrophils

Whole blood was obtained from 10 mice. Heparinized blood was mixed with an equal volume of 3% dextran in 0.9% NaCl. After 30 minute of gravity sedimentation, the upper layer, containing leukocytes, was removed and centrifuged at 620 g for 10 minute. The cell pellet was resuspended in 0.9% NaCl and underlaid with Ficoll-Paque (GE Healthcare, Tokyo, Japan). After centrifugation for 30 minute at 1,490 g, the mononuclear cell layer was isolated and contaminating red blood cells were removed by hypotonic lysis. After centrifugation for 10 minute at 760 g (two times), the pellet was resuspended and the neutrophils were plated at 1.0×10^6 cells/mL in PBS.

Measurement of neutrophil-derived reactive oxygen species

The scavenging activity of HSA-Trx against $O_2^{\cdot-}$ released from neutrophils was determined using electron paramagnetic resonance (EPR) spin trapping with DMPO. The neutrophils (1.0×10^6 cells/ml) were pre-treated with PMA (1 μg/ml) for 7 minute at 37ºC to activate the cells and generate ROS. Aliquots of this cell suspension were combined with 100 μM DTPA in HBSS in the absence or presence of either HSA-Trx (10, 30, 50 μM), HSA (30 μM) or Trx (30 μM). After

activation of neutrophils, this reaction mixture was added with DMPO (27 mM). After 6 minute from addition of DMPO, EPR spectra were recorded at room temperature in a JES-TE-200 spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.43 GHz; microwave power, 40 mW; scanning field, 335.2 ± 5 mT; sweep time, 2 min; field modulation width, 0.25 mT; receiver gain, 630; and time count, 0.3 s.

Radiolabeling of proteins with ^{125}I

HSA-Trx and Trx were radiolabeled with ^{125}I according to the procedures reported previously (Furukawa et al., 2011; Watanabe et al., 2001), and purified using a Pharmacia Bio-Gel PD-10 column. The radiolabeled proteins were diluted with non-labeled protein before conducting the pharmacokinetic experiments to adjust the dose (mg/kg) of protein in each group.

Pharmacokinetics of Trx and HSA-Trx in BLM-mice

On day 14 after BLM administration, the ^{125}I-proteins (0.1 mg/kg) were injected into the tail vein of mice (about 10^5 cpm/mice). Approximately 500 μl of blood samples was collected from the vena cava at 0.05, 0.167, 0.5, 1, 2, 4, 6, and 12 hour after the injection of these radiolabeled proteins with the mice under ether anesthesia, and plasma was obtained. Degraded proteins and free ^{125}I were removed from plasma by centrifugation in 1% bovine serum albumin and 40% TCA. At each of these time points, the mice were sacrificed. The organs were rinsed with saline and weighed, and the ^{125}I radioactivity contained in each tissue was determined using a γ-counter (ARC-5000; Aloka).

Data analysis

Non-compartment model was used in the pharmacokinetic analyses after ^{125}I-proteins administration. Each parameter was calculated by using a program MOMENT (BS) (Tabata et al., 1996). Data are means ± SD for the indicated number of animals. Significant differences among each group were examined using a one-way of analysis of variance (ANOVA) followed by Tukey

multiple comparison. A probability value of $p< 0.05$ was considered to indicate statistical significance.

RESULTS

Evaluation of the administration schedule of HSA-Trx in BLM-induced pulmonary fibrosis

Pulmonary fibrosis was induced in mice by a single intratracheal administration of BLM (day 0). Since Hoshino et al found that the multiple administration of Trx (3.5 nmol/body) prevented BLM-induced pulmonary fibrosis (Hoshino et al., 2003), we adopted a dose of HSA-Trx equivalent to one Trx treatment (3.5 nmol/body). Fig. 1 shows a schematic summary of the experimental protocols used in the study. The effect of HSA-Trx on BLM-induced pulmonary fibrosis was evaluated by HE staining (Fig. 2a), Masson's trichrome staining (Fig. 2b), the quantity analysis of the section positively stained for collagen (Fig. 2c) and the measurement of hydroxyproline levels in lung tissue (Fig. 2d) on day 14.

HE staining showed that BLM administration induced severe lung damage (thickened and edematous alveolar walls and interstitia) and the infiltration of inflammatory cells into these regions (Fig. 2a). Masson's trichrome staining of collagen and the measurement of hydroxyproline levels in lung tissue indicated that BLM induced the deposition of collagen in lung (Fig. 2b, c and d). These phenomena induced by BLM were all significantly suppressed by the HSA-Trx treatment. In addition, the administration of HSA-Trx at weekly intervals had a more potent effect than its use at 2 week intervals (Fig. 2a-d). Therefore, HSA-Trx was administered once per week in the following experiments.

Effect of HSA-Trx on histological alterations and hydroxyproline levels in lung tissue

Hoshino et al demonstrated that when Trx is administered every 2 days, it prevented BLM-induced pulmonary fibrosis (Hoshino et al., 2003). Thus, we compared the effect of the intravenous administration of HSA-Trx at weekly intervals with that for the intravenous of Trx at weekly intervals, or the intraperitoneal administration of Trx at 2 day intervals (Fig. 1b).

HE staining (Fig. 3a), Masson's trichrome staining (Fig. 3b), the quantity analysis of the section positively stained for collagen (Fig. 3c) and hydroxyproline levels in lung tissue (Fig. 3d) clearly

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indicated that HSA-Trx, when administered at 1 week intervals, significantly suppressed the lung injury and fibrosis in diseased model mice. The *ip* administration of Trx at 2 day intervals resulted in similar therapeutic effects as those for the HSA-Trx treatment at one week intervals, but the *iv* administration of Trx at one week intervals showed no suppressive effect.

Effect of HSA-Trx on active TGF-β1 levels in lung tissue

TGF-β1 plays an important role in BLM-induced pulmonary fibrosis (Chaudhary et al., 2006; Kinnula et al., 2005). To reveal the mechanism underlying the suppressive effect of HSA-Trx on BLM-induced pulmonary fibrosis, the levels of active TGF-β1 in lung tissue on day 7 were determined. As shown in Fig. 4, the level of active TGF-β1 was increased in BLM-mice (BLM (+), saline (*iv*)). In contrast, HSA-Trx decreased the level of active TGF-β1 to the same level as the normal group (BLM (-)).

Effect of HSA-Trx on BALF cells

To evaluate the effects of HSA-Trx on the inflammatory response induced by BLM, the cells in BALF were analyzed. As shown in Fig. 5, the administration of BLM resulted in an increase in the number of inflammatory cells (total cells: Fig. 5a), including alveolar macrophages (Fig. 5b), neutrophils (Fig. 5c) and lymphocytes (Fig. 5d) on days 1 and 3 after BLM administration. The HSA-Trx treatment significantly reduced the number of total cells and neutrophils on both days 1 and 3, and alveolar macrophages and lymphocytes on day 3. These results suggest that HSA-Trx ameliorates the BLM-induced pulmonary inflammatory response.

Effect of HSA-Trx on inflammatory cytokines and chemokine levels in lung tissue

We also examined the effect of HSA-Trx on IL-6, TNF-α and MIF levels in the lung tissue of BLM-induced pulmonary fibrosis on days 3 and 7. As shown in Fig. 6, the levels of IL-6 (Fig. 6a), TNF-α (Fig. 6b) and MIF (Fig. 6c and d) in lung tissue that were increased by BLM were

significantly decreased as the result of the HSA-Trx treatment. These data suggest that HSA-Trx exerts an anti-inflammatory action against BLM-induced pulmonary damage.

Effect of HSA-Trx on oxidative stress in lung tissue

Recently reported findings suggest that reactive oxygen species (ROS) released from activated leukocytes, especially alveolar macrophages and neutrophils, are associated with the development of BLM-induced lung injury (Manoury et al., 2005). To evaluate the effect of HSA-Trx on the oxidative stress induced by BLM in the lung, immunostaining of 8-OH-dG and NO_2-Tyr, an oxidized product of nucleic acids and proteins, respectively, and also quantification of lipoperoxidation final reaction substances, malondialdehyde (MDA) in lung sections were performed on day 3 after BLM administration. As shown in Fig. 7, the accumulation of 8-OH-dG, NO_2-Tyr and MDA in lung tissue increased in BLM-mice (BLM (+), saline (*iv*)) as compared to normal mice (BLM (-)), while HSA-Trx clearly suppressed the levels of these oxidative stress markers in the lungs.

To confirm whether HSA-Trx shows scavenging activity against $O_2^{\cdot-}$ generated from neutrophils, we conducted ex vivo EPR spectroscopy using a DMPO spin trapping technique. As shown in Fig. 8, although PMA-stimulated neutrophils generated $O_2^{\cdot-}$ and increased EPR signaling, HSA-Trx significantly decreased the EPR signaling in a concentration dependent manner (Fig. 8a and b). We also compared the $O_2^{\cdot-}$ scavenging activity of HSA-Trx, Trx and HSA. At a 30 μM concentration of each protein, HSA-Trx and Trx significantly decreased the EPR signal by 40% and 85%, respectively, while HSA alone did not significantly change the intensity of the signal (Fig. 8c and d).

Effect of post-treatment of HSA-Trx on BLM-induced pulmonary fibrosis

For future clinical applications, the post-administration effect of HSA-Trx against BLM-induced fibrosis was examined (Fig. 1c). Since HSA-Trx suppressed ROS production by neutrophils (Fig. 8),

the effect of the post-administration of HSA-Trx was examined at 1 and 7 days after BLM treatment when a marked increase in neutrophils in BALF was observed (Fig. 5c). The results of HE staining (Fig. 9a), Masson's trichrome staining (Fig. 9b), the quantity analysis of the section positively stained for collagen (Fig. 9c) and the determination of hydroxyproline levels (Fig. 9d) on day 14 showed that the post-treatment of HSA-Trx suppressed the progression of BLM-induced fibrosis.

Pharmacokinetics of HSA-Trx in BLM mice

A pharmacokinetics study of ^{125}I-labeled HSA-Trx or Trx was carried out using BLM-mice. As shown in Fig. 10, no significant difference between the plasma concentration-time profiles for HSA-Trx in BLM-mice (BLM (+)) and that in normal mice (BLM (-)) were found. The half-life of HSA-Trx was about 8 hour in both BLM (8.45 ± 0.85 hr) and normal (7.89 ± 0.23 hr) mice, which is more than 10 times longer than that of Trx (0.50 ± 0.02 hr) in the model mice (Fig. 10a). In addition, no significant difference was observed in the tissue distribution patterns of the HSA-Trx in BLM and normal mice, where the highest concentration was found in the plasma, followed by the kidney, lung, spleen and liver (Fig. 10b). On the other hand, the tissue distribution of Trx in the kidneys was the highest in BLM-mice (Fig. 10b). These pharmacokinetic properties of HSA-Trx or Trx alone were similar to previously reported results using normal and OVA-induced lung injury mice (Ikuta et al., 2010; Furukawa et al., 2011).

DISCUSSION

IPF is a refractory lung disorder for which no effective treatment is available. Trx was recently shown to be present at higher concentrations in the sera and lungs of IPF patients, compared to healthy subjects (Iwata et al., 2010). In addition, *in vivo* and *in vitro* investigations have shown that BLM treatment caused Trx to be expressed at high levels in bronchial epithelial cells, although no other endogenous anti-oxidative proteins, i.e., Cu/Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase, were induced (Gon et al., 2001). These collective findings suggest that Trx may play an important role in the protection of lungs in IPF patients. In this study, we evaluated the therapeutic effects of HSA-Trx on mice with BLM-induced pulmonary fibrosis, and obtained four important findings. 1) HSA-Trx suppressed the progression of pulmonary fibrosis when given once-a-week regimen. 2) The mechanism by which HSA-Trx inhibits lung damage induced by BLM can be attributed to the long acting anti-oxidative and anti-inflammatory modulation effects of Trx. 3) The post-administration of HSA-Trx was effective in suppressing lung damage. 4) In the BLM-induced pulmonary fibrosis animal model, HSA-Trx exhibited similar pharmacokinetic properties to that in healthy animals e.g. increased blood retention and enhanced distribution to the lungs.

Because the BLM-induced lung disorder animal model used in this study manifested lung disorders with a greater severity than other conventional pathologic models, it is possible that the pathological conditions contributed to the changes in the pharmacokinetics of HSA-Trx. However, the administration of BLM and the subsequent pulmonary fibrosis did not change the pharmacokinetic behavior of HSA-Trx (Fig. 10). This observation led to assumption that HSA-Trx could be used as a long-acting Trx that will persistently exhibit a lung-protective action in the BLM-induced lung disorders animal model. In fact, consistent with the results reported by Hoshino et al., 2003, we found that the intravenous administration of Trx at weekly intervals did not prevent pulmonary fibrosis due to its short half life, whereas the once-a-week intravenous administration of HSA-Trx suppressed the progression of fibrosis to the same extent as the intraperitoneal administration of Trx at 2 day intervals (Fig. 3). Such a reduction in the dose-frequency of Trx by

fusing the protein to HSA is a favorable outcome from the view point of clinical applications. To the best of our knowledge, this is the first demonstration of HSA-Trx being used as an effective long-acting therapeutic agent against BLM-induced lung disordered models.

BLM-induced pulmonary fibrosis is a pathological condition in which ROS is produced at an early stage of BLM treatment, serves as a key trigger to cause the further production of ROS that, in turn, promotes inflammatory cell infiltration and the associated secretion of inflammatory cytokines, resulting in the progression of lung injury and inflammation, and eventually fibrosis. The suppressive effect of HSA-Trx on BLM-induced pulmonary fibrosis could be due to the anti-oxidative activity of Trx, since Trx exhibits superior anti-oxidative characteristics via a redox reaction of SH and S-S at its active site (Holmgren, 1989). In fact, we found that HSA-Trx significantly suppressed the accumulation of oxidative stress markers in the lungs (8-OH-dG, NO_2-Tyr and MDA) at day 3 of a BLM treatment (Fig. 7). Another investigation involving EPR experiments using a DMPO spin trapping agent examined the direct scavenging capacity of HSA-Trx for neutrophil-derived ROS, revealing the concentration-dependent suppression of $O_2^{\cdot-}$ production by HSA-Trx (Fig. 8).

Inflammatory cells such as activated leukocytes, especially neutrophils, are considered to be major contributors to the onset and progression of IPF and BLM-induced pulmonary fibrosis due to their dramatic increases at the beginning of inflammation (Fig. 5c) and subsequent release of a large amount of $O_2^{\cdot-}$ (Kinder et al., 2008; Manoury et al., 2005). A comparison of the neutrophil-produced $O_2^{\cdot-}$ scavenging capacities of HSA-Trx and Trx showed that HSA-Trx retained about 50% of the specific activity of Trx (Fig. 8). This reduced activity of HSA-Trx is likely attributable to microenvironmental changes in the active center of Trx or steric hindrance as the result of the formation of a fusion complex (Muller et al., 2007).

TGF-β1 promotes interstitial collagen production via fibroblast activation and epithelial cell transition to mesenchymal cells (epithelial-mesenchymal transition; EMT) (Willis and Borok, 2007). A recent in vitro study showed that ROS and inflammatory cytokines promoted TGF-β1 production

by pulmonary epithelial cells and its activation (Barcellos-Hoff and Dix, 1996; Bellocp et al., 1999). Since HSA-Trx scavenges ROS, it should be able to suppress the production of active TGF-β1 in lung tissue. The findings reported herein indicate that HSA-Trx significantly reduced the active TGF-β1 content in lung tissue induced by the BLM treatment (Fig. 4). Hence, the results suggest that the suppression of ROS production and inflammatory cell infiltration at an early-stage of BLM treatment by HSA-Trx eventually led to the suppression of active TGF-β1 production.

Active TGF-β1, in turns, activates NADPH oxidase in fibroblasts thereby enhances ROS production and downregulates the expression of glutamate cysteine ligase (Arsalane et al., 1997; Thannickal and Fanburg, 1995), resulting in aggravation of the redox balance in the body. It is therefore necessary to block the vicious circle of oxidative stress caused by active TGF-β1 in order to suppress BLM-induced pulmonary fibrosis. Since a weekly administration of HSA-Trx produced a better suppressing effect on pulmonary fibrosis than the fortnightly administration of HSA-Trx (Fig. 2), the ROS-scavenging action of HSA-Trx administered after the 7 day of BLM treatment may inhibit this vicious circle.

In addition to its anti-oxidative action, Trx also has anti-inflammatory characteristics, including its ability to suppress the migration/infiltration and extravascular leakage of inflammatory cells (Nakamura et al., 2001). Therefore, it is highly possible that the anti-inflammatory actions of Trx are involved in the therapeutic effects of HSA-Trx against BLM-induced lung injury. In fact, HSA-Trx suppressed the pulmonary infiltration of inflammatory cells at days 1 and 3 of the administration of BLM (Fig. 5). The anti-inflammatory effects of Trx can be attributed mainly to suppression of the production of cytokines and chemokines. We also found that HSA-Trx significantly lowered the lung concentrations of these cytokines in mice with BLM-induced lung disorders (Fig. 6a and b). While the mechanism of suppression by Trx on the production of these cytokines remains somewhat unclear, an interesting observation was recently reported that Trx exhibited its anti-inflammatory effect by suppressing the expression and activity of MIF (Son et al., 2009; Tamaki et al., 2006). MIF promotes the production of TNF-α, IL-6, and other cytokines, as

well as ROS, such as nitric oxide, superoxide anions etc (Baugh and Bucala, 2002). MIF is expressed at increased levels in a broad range of oxidative stress situations and inflammation related pathological conditions such as sepsis, rheumatoid arthritis, inflammatory bowel disease, and lung disorders including BLM-induced pulmonary fibrosis (Tanino et al., 2002). Furthermore, the administration of a MIF-neutralizing antibody has been shown to improve survival rates by significantly suppressing the pulmonary infiltration of inflammatory cells and the development of lung disorders (Tanino et al., 2002). Interestingly, HSA-Trx was also able to significantly suppress MIF expression induced by BLM administration (Fig. 6c and d). Taking all these findings into consideration, the inactivation of MIF by HSA-Trx may involve the anti-inflammatory effects of HSA-Trx observed in this study as well.

The findings of this study indicate that the progression of lung injury in a BLM-induced lung disorder mice model could be suppressed by the pre- or post-administration of HSA-Trx, demonstrating its potential as a novel therapeutic agent for the treatment of IPF, acute interstitial pneumonia or drug-induced lung diseases. HSA-Trx may function as a long acting anti-oxidative and anti-inflammatory modulator. In recent years, acute exacerbation of IPF due to a cold, influenza, etc., along with its progression, have contributed to the mortality of IPF patients, and preventing acute exacerbation has been a key strategy for the treatment of the disease. Because HSA-Trx suppressed lung disorders, even when administered after BLM treatment, it is speculated that HSA-Trx may be effective in the prevention and treatment of the acute exacerbation associated with IPF. While HSA-Trx exhibited similar blood retention levels as HSA in the BLM-induced lung disorders animal model, it is speculated to have a therapeutic effect in IPF patients with a possibility of fortnightly or monthly administrations, considering the fact that the half-life of HSA in humans is approximately 20 day (Peters, 1985). Although further investigations related to establishing the dosing regimen need to be carried out, a reduced dosing frequency and stable therapeutic effect of HSA-Trx would make the treatment more convenient, safe, cost effective and improve the health-related quality of life for IPF patients. The BLM animal model is widely used in the

assessment of potential antifibrotic agents. However, the aspect of slow and irreversible progression of IPF in patients is not reproduced in the BLM model. It would be interesting to see whether this therapeutic could reverse or slow progression of fibrosis in clinical setting as opposed to preventing the response evoked by BLM.

Authorship Contributions

Participated in research design: Tanaka, Watanabe, Kodama, Chuang, Ishima, Hamasaki, Tanaka, Mizushima, Otagiri, and Maruyama

Conducted experiments: Tanaka, Watanabe, Kodama, Ishima

Contributed new reagents or analytic tool: Tanaka, Watanabe, Kodama

Performed data analysis: Tanaka, Watanabe, Kodama

Wrote or contributed to writing of the manuscript: Tanaka, Watanabe, Chuang, Otagiri, and Maruyama

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Footnotes

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Figure legends

Fig. 1. Schematic summary of the experimental protocol for evaluating the effect of HSA-Trx on BLM-induced pulmonary fibrosis used in the study: HSA-Trx was administrated intravenously at day 0 and day 7 (refer to "every 1 week", Fig. 1a), or only day 0 (refer to "every 2 weeks", Fig. 1a). On day 0, HSA-Trx was administered 30 minute before BLM treatment. For the intervention study, the first dose of HSA-Trx was administered 1 day after BLM treatment, and the subsequent dose administered at day 7 after BLM treatment (Fig. 1c).

Fig. 2. Effect of HSA-Trx (*i.v.*) (every 2 weeks or every 1 week) on the BLM-induced pulmonary fibrosis: (a), (b) Sections of pulmonary tissue were prepared 14 day after BLM administration, and subjected to histopathological examination [(a) HE and (b) Masson's trichrome staining]. (c) Fibrosis score was evaluated as the quantity of the section positively stained for collagen and displaying alveolar wall thickening. (d) Hydroxyproline levels in lung were determined 14 day after BLM administration. Each bar represents the mean±SD (c: n=3~5, d: n=5).

Fig. 3. Effects of HSA-Trx (*i.v.*) (every 1 week), Trx (*i.v.*) (every 1 week) or Trx (*i.p.*) (every 2 day) on the BLM-induced pulmonary fibrosis: (a), (b) Sections of pulmonary tissue were prepared 14 day after BLM administration, and subjected to histopathological examination [(a) HE and (b) Masson's trichrome staining]. (c) Fibrosis score was evaluated as the quantity of the section positively stained for collagen and displaying alveolar wall thickening. (d) Hydroxyproline levels in lung were determined 14 day after BLM administration. Each bar represents the mean±SD (c: n=4, d: n=5). $^{**}p<0.01$ or $^{*}p<0.05$ vs BLM (+), Saline (*i.v.*).

Fig. 4. Effect of HSA-Trx on active TGF-β1 levels in BLM mice lung: Active TGF-β1 levels in lung were determined 7 day after BLM administration. Each bar represents the mean±SD (n = 5).

Fig. 5. Effect of HSA-Trx on BALF cells in BLM mice: Inflammatory cells number including (a) total cells, (b) alveolar macrophages, (c) neutrophils and (d) lymphocytes was determined 1 day and 3 day after BLM administration. Each bar represents the mean±SD (n = 6). ***p*<0.01 vs BLM (-).

Fig. 6. Effect of HSA-Trx on pulmonary inflammatory cytokines and chemokine in BLM mice: (a) IL-6 levels and (b) TNF-α levels in lung were determined and (c, d) MIF expression in lung were confirmed and analyzed 3 and 7 days after BLM administration. Each bar represents the mean±SD (a and b: n=5~6, d: n=3~4). ***p*<0.01 or **p*<0.05 vs BLM (-).

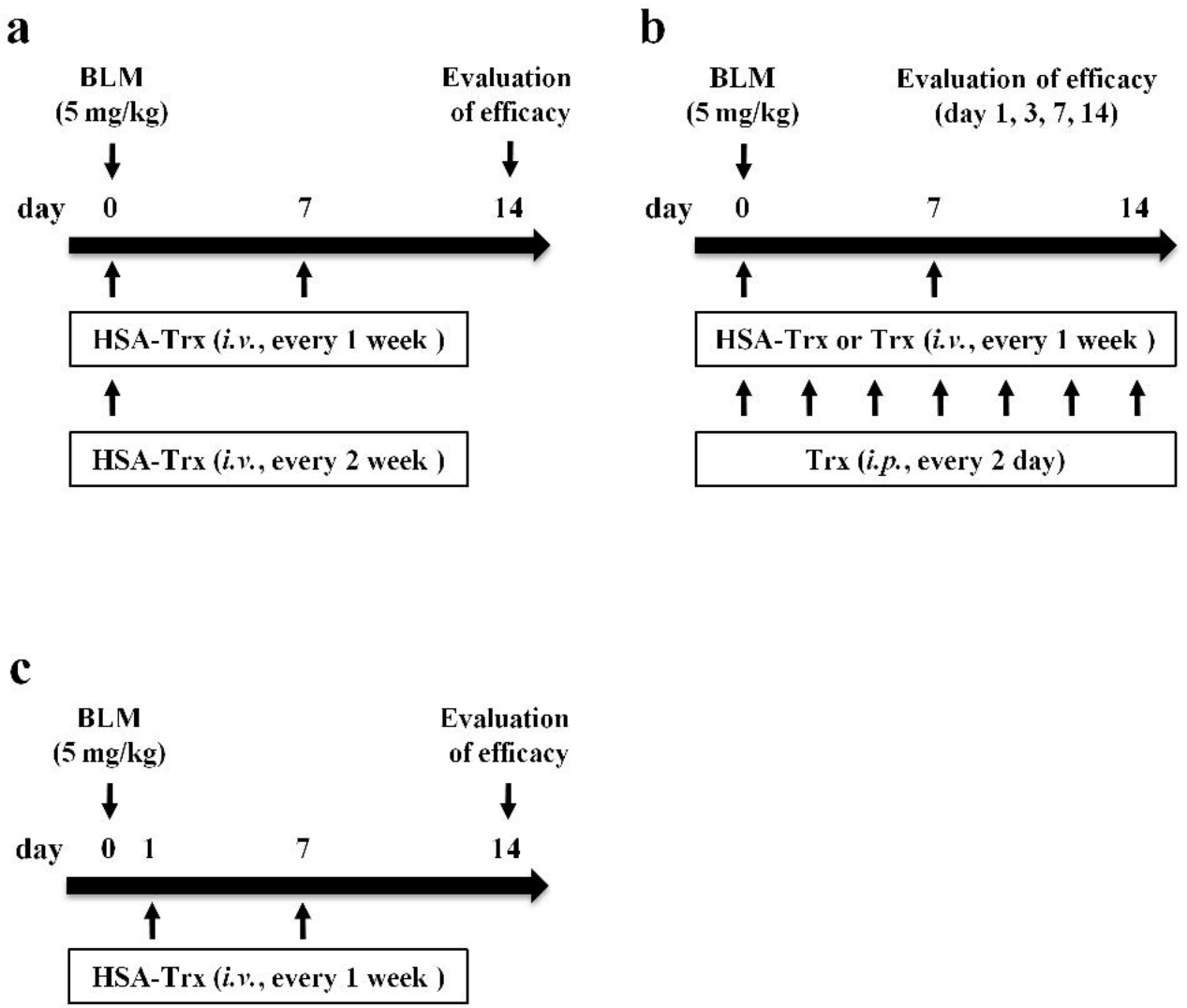
Fig. 7. Effect of HSA-Trx on pulmonary oxidative stress in BLM mice: The immunostaining of the frozen lungs slice for the oxidative stress markers of nucleic acid, (a) 8-OH-dG and amino acid, (b) NO_2-Tyr were performed 3 day after BLM administration. Image analysis of the extent and intensity of (c) 8-OH-dG and (d) NO_2-Tyr staining was performed. (e) The MDA levels in lung were determined 3 day after BLM administration. Each bar represents the mean±SD (c and d: n=4, e: n=6).

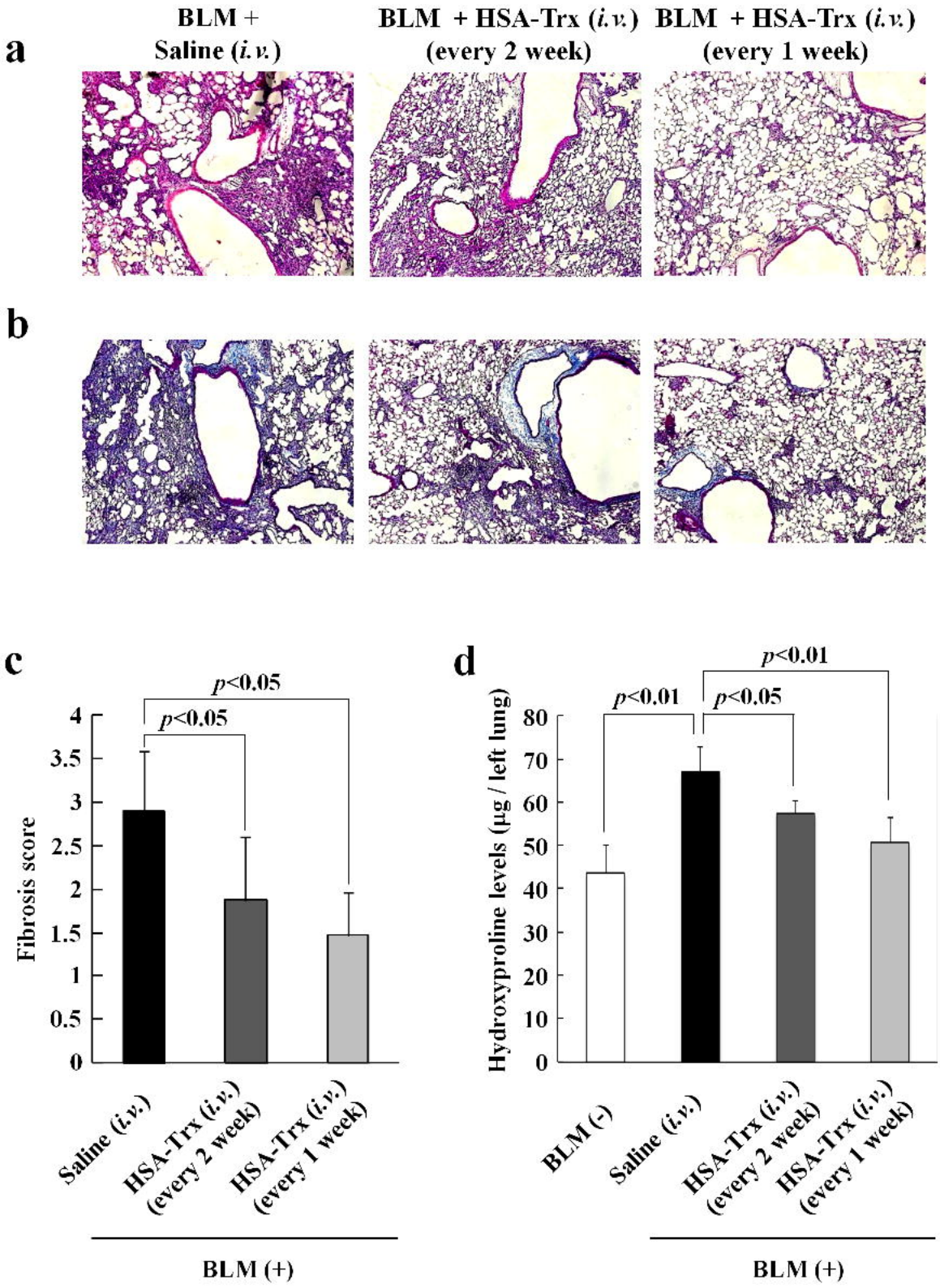
Fig. 8. Effect of HSA-Trx on neutrophil-derived ROS using EPR spectroscopy: The concentration dependent scavenging activity of HSA-Trx against ROS released from neutrophils was determined using EPR spin trapping with DMPO. (a) EPR spectrum of DMPO spin adducts of $^{\cdot}HO$ and (b) the quantitation of the $^{\cdot}HO$ concentration were shown. The scavenging activity of HSA-Trx, HSA and Trx (30 μM) against ROS released from neutrophils was determined by EPR spin trapping with DMPO. (c) EPR spectrum of DMPO spin adducts of $^{\cdot}HO$ and (d) the quantitation of the $^{\cdot}HO$ concentration were shown. Each bar represents the mean±SD (n = 3). ***p*<0.01 vs HBSS or $^{\#\#}p$<0.01 vs Trx.

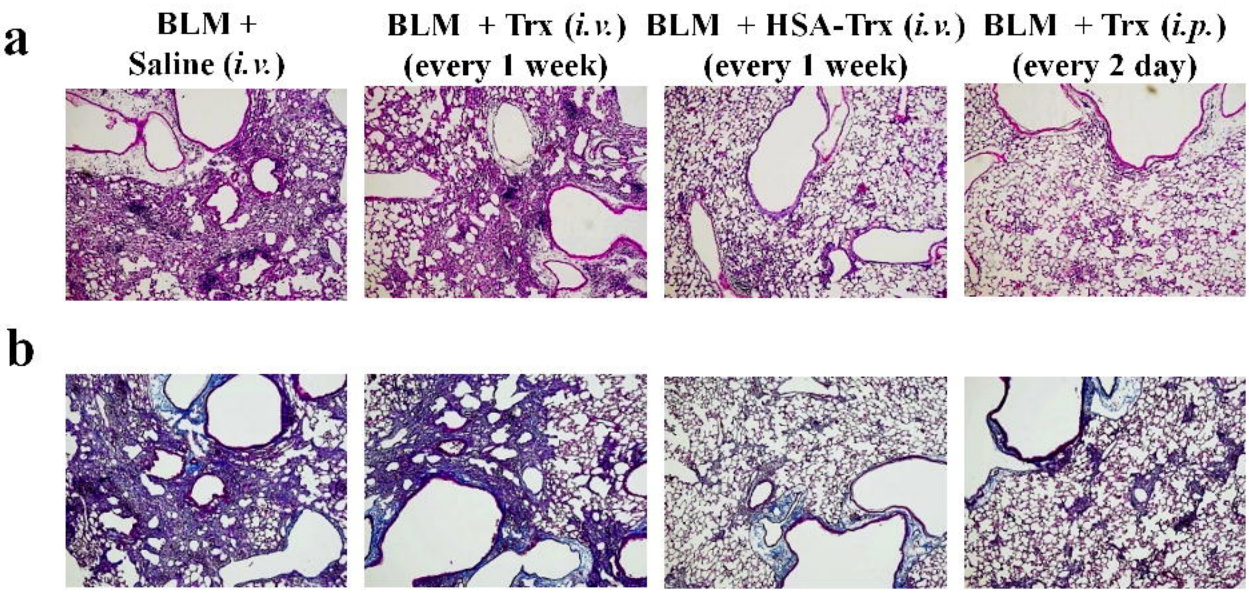
Fig. 9. Effects of post-administration of HSA-Trx (every 1 week) against BLM-induced

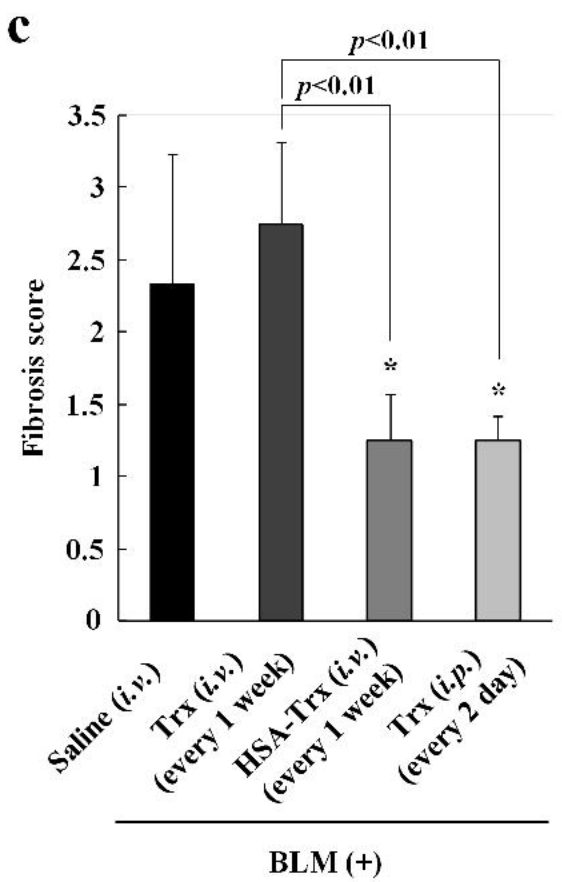
pulmonary fibrosis 1 day after BLM treatment: (a), (b) Sections of pulmonary tissue were prepared 14 day after BLM administration, and subjected to histopathological examination [(a) HE and (b) Masson's trichrome staining]. (c) Fibrosis score was evaluated as the quantity of the section positively stained for collagen and displaying alveolar wall thickening. (d) Hydroxyproline levels in lung were determined 14 day after BLM administration. Each bar represents the mean±SD (c: n=4, d: n=5).

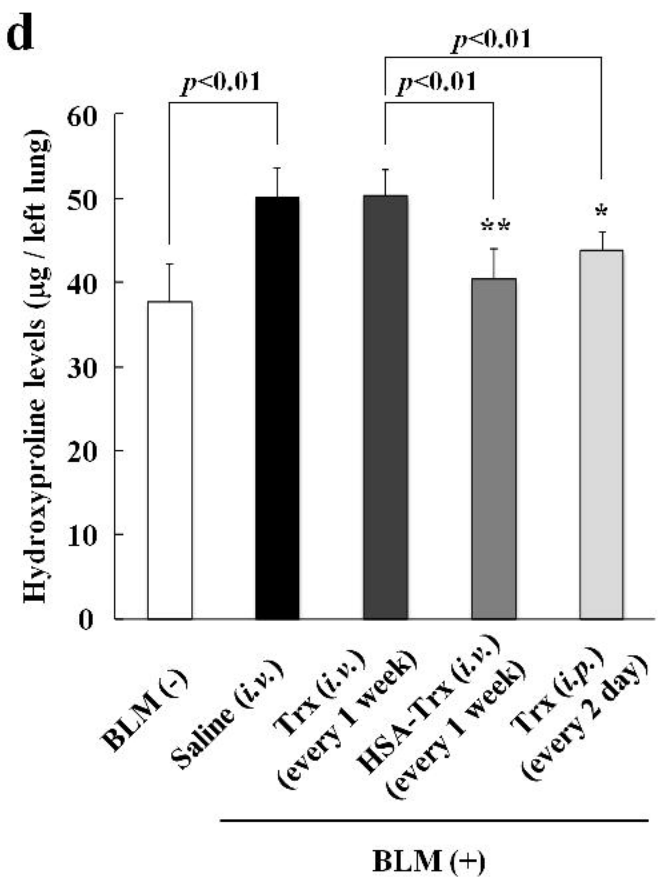
Fig. 10. Pharmacokinetic profiles of HSA-Trx and Trx and in BLM-mice: (a) Plasma levels of ^{125}I-labeled HSA-Trx and Trx after intravenous administration into the tail vein of BLM and normal mice. Each point represents mean±SD (n=3) ; (b) Tissue distribution profile of HSA-Trx and Trx in kidney, liver, lung and spleen at 2 hour after intravenous administration into the tail vein of BLM and normal mice. Each bar represents the mean±SD (n = 3). $^{**}p<0.01$ vs Trx (BLM (+)).

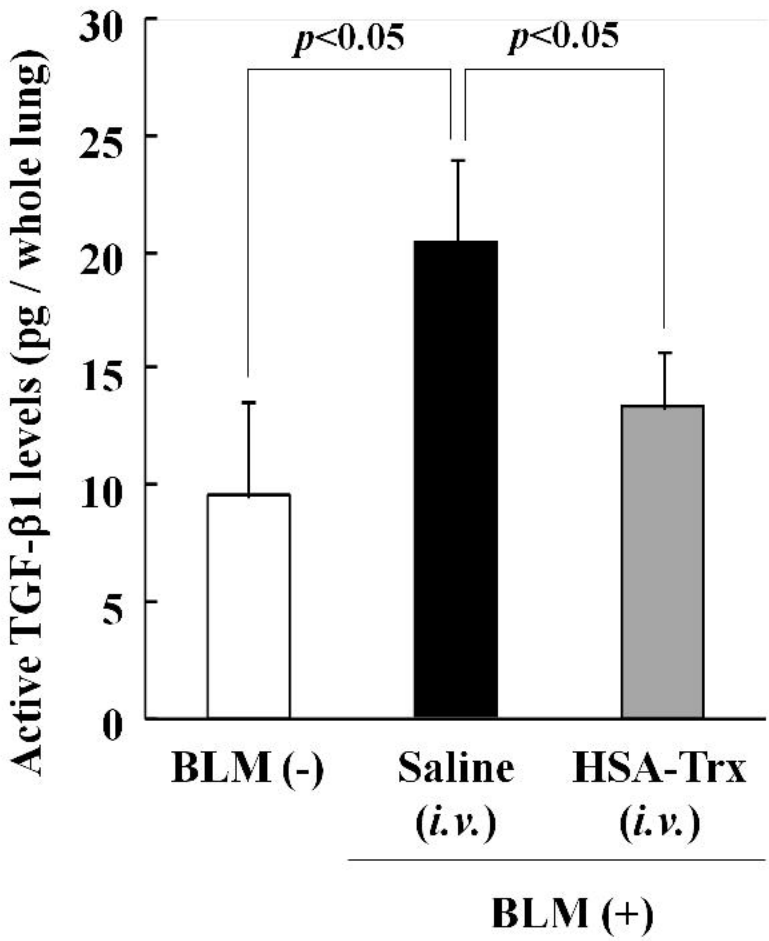

a
BLM
(5 mg/kg)
Evaluation
of efficacy
day 0 7 14
HSA-Trx (i.v., every 1 week)
HSA-Trx (i.v., every 2 week)
b
BLM
(5 mg/kg)
Evaluation of efficacy
(day 1, 3, 7, 14)
day 0 7 14
HSA-Trx or Trx (i.v., every 1 week)
Trx (i.p., every 2 day)
c
BLM
(5 mg/kg)
Evaluation
of efficacy
day 0 1 7 14
HSA-Trx (i.v., every 1 week)

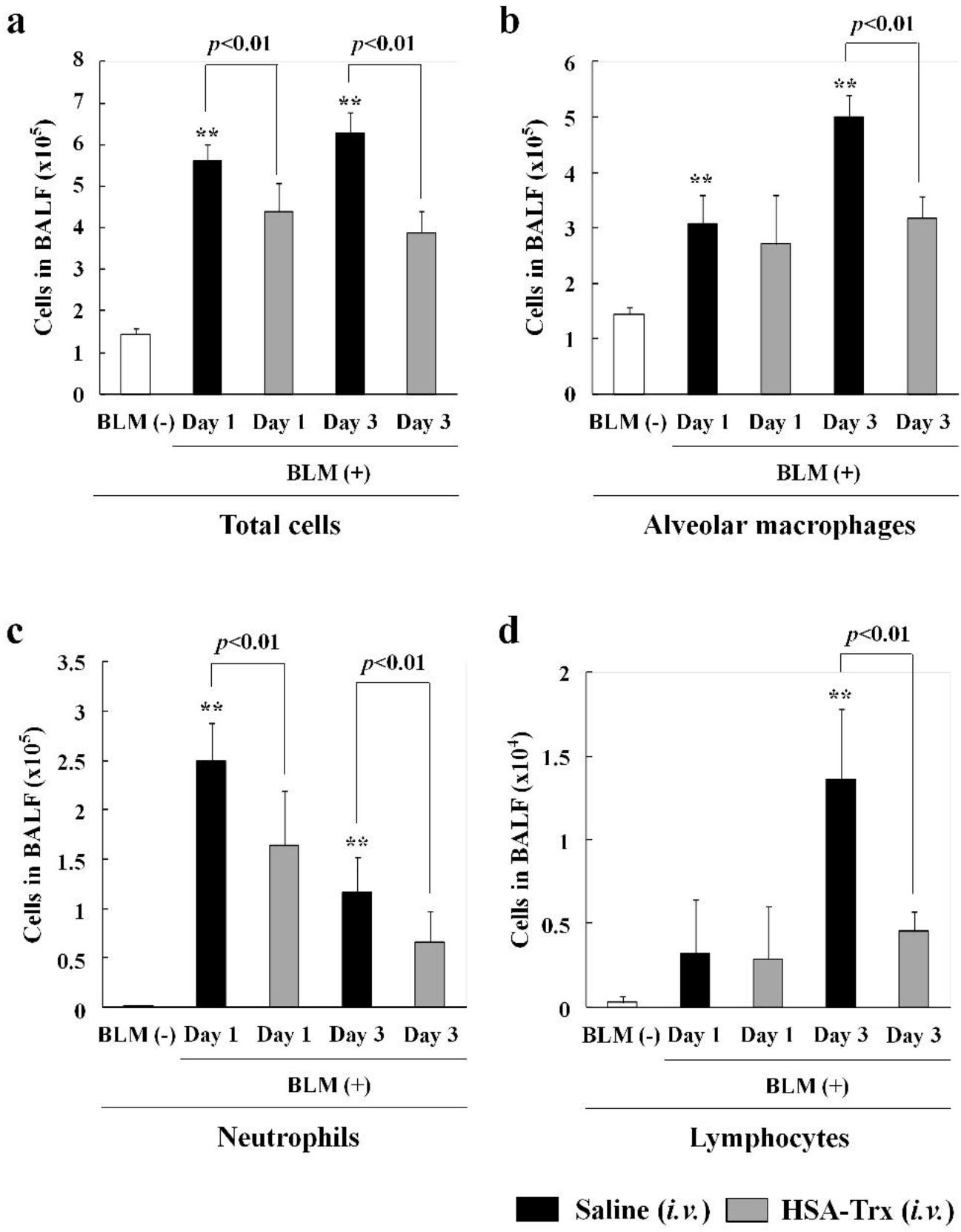

a
BLM + Saline (i.v.)
BLM + HSA-Trx (i.v.) (every 2 week)
BLM + HSA-Trx (i.v.) (every 1 week)
b
c
p<0.05
p<0.05
Fibrosis score
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline (i.v.)
HSA-Trx (i.v.) (every 2 week)
HSA-Trx (i.v.) (every 1 week)
BLM (+)
d
p<0.01
p<0.01
p<0.05
Hydroxyproline levels (μg / left lung)
80
70
60
50
40
30
20
10
0
BLM (-)
Saline (i.v.)
HSA-Trx (i.v.) (every 2 week)
HSA-Trx (i.v.) (every 1 week)
BLM (+)

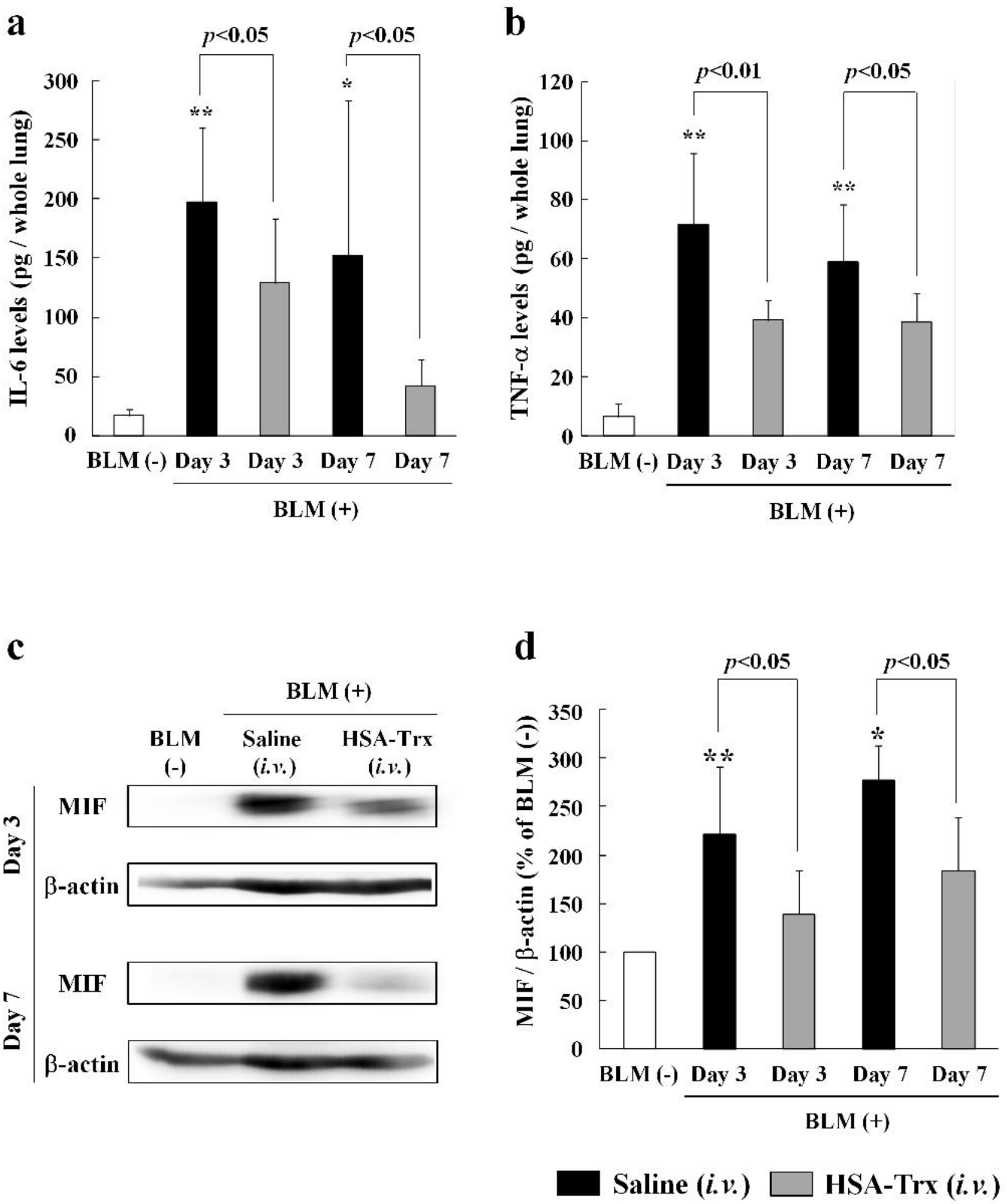

a
BLM + Saline (i.v.)
BLM + Trx (i.v.) (every 1 week)
BLM + HSA-Trx (i.v.) (every 1 week)
BLM + Trx (i.p.) (every 2 day)
b

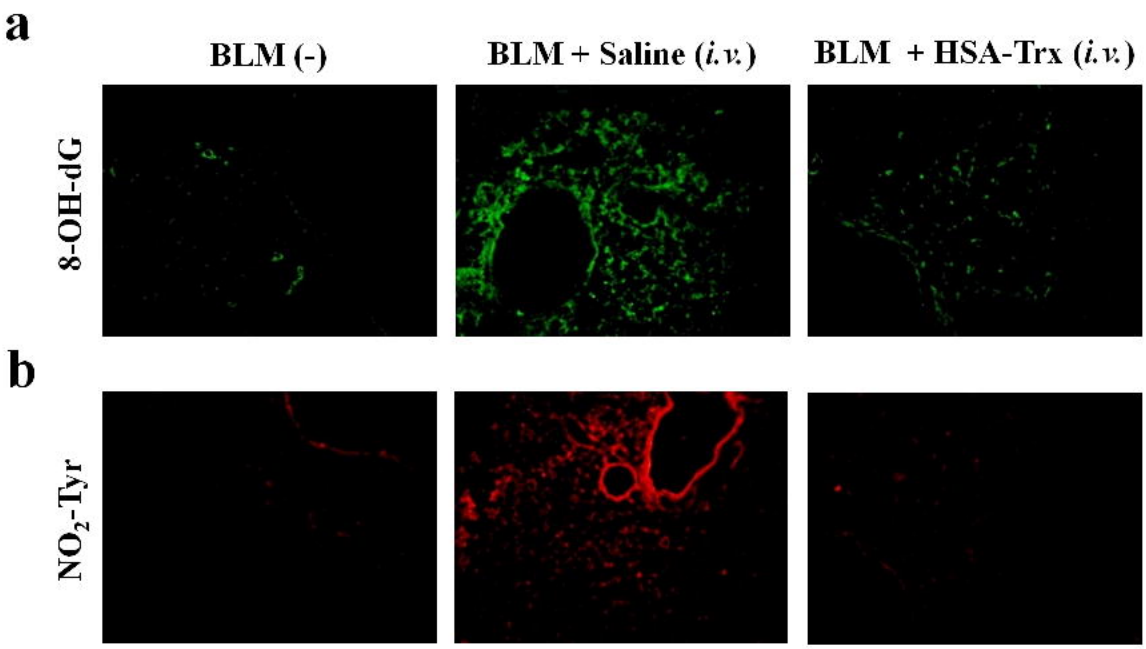

c
p<0.01
p<0.01
Fibrosis score
3.5
3
2.5
2
1.5
1
0.5
0
*
*
Saline (i.v.)
Trx (i.v.) (every 1 week)
HSA-Trx (i.v.) (every 1 week)
Trx (i.p.) (every 2 day)
BLM (+)

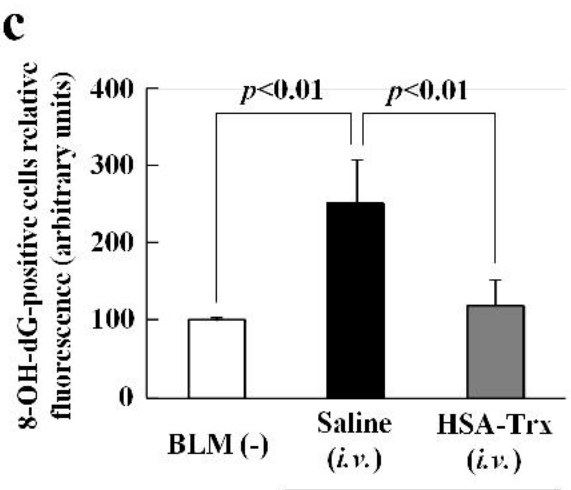

d
p<0.01
p<0.01
p<0.01
Hydroxyproline levels (μg / left lung)
60
50
40
30
20
10
0
**
*
BLM (-)
Saline (i.v.)
Trx (i.v.) (every 1 week)
HSA-Trx (i.v.) (every 1 week)
Trx (i.p.) (every 2 day)
BLM (+)

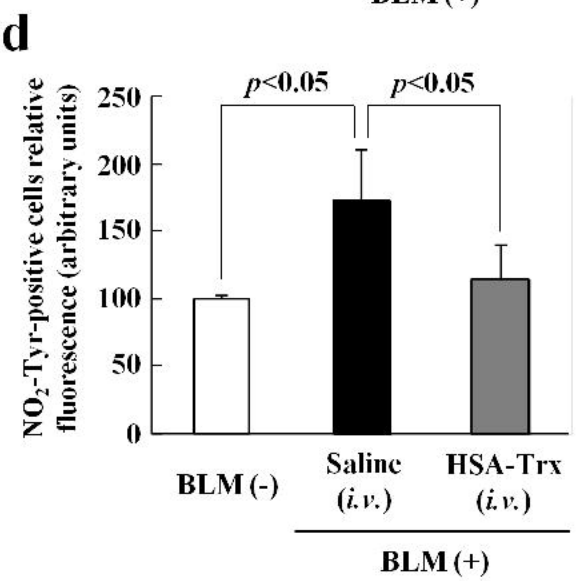

30
25
20
15
10
5
0
Active TGF-β1 levels (pg / whole lung)
p<0.05
p<0.05
BLM (-)
Saline
(i.v.)
HSA-Trx
(i.v.)
BLM (+)

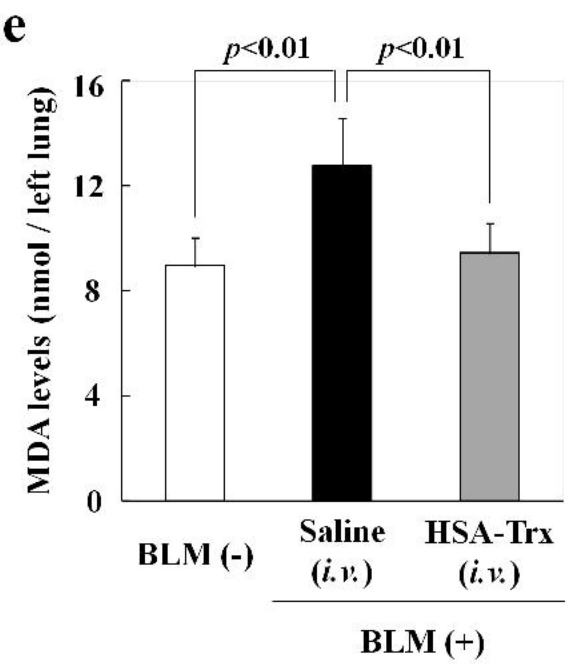

a
p<0.01
p<0.01
**
**
Cells in BALF (x10^5)
8
7
6
5
4
3
2
1
0
BLM (-) Day 1 Day 1 Day 3 Day 3
BLM (+)
Total cells
b
p<0.01
**
**
Cells in BALF (x10^5)
6
5
4
3
2
1
0
BLM (-) Day 1 Day 1 Day 3 Day 3
BLM (+)
Alveolar macrophages
c
p<0.01
p<0.01
**
**
Cells in BALF (x10^5)
3.5
3
2.5
2
1.5
1
0.5
0
BLM (-) Day 1 Day 1 Day 3 Day 3
BLM (+)
Neutrophils
d
p<0.01
**
Cells in BALF (x10^4)
2
1.5
1
0.5
0
BLM (-) Day 1 Day 1 Day 3 Day 3
BLM (+)
Lymphocytes
Saline (i.v.)
HSA-Trx (i.v.)

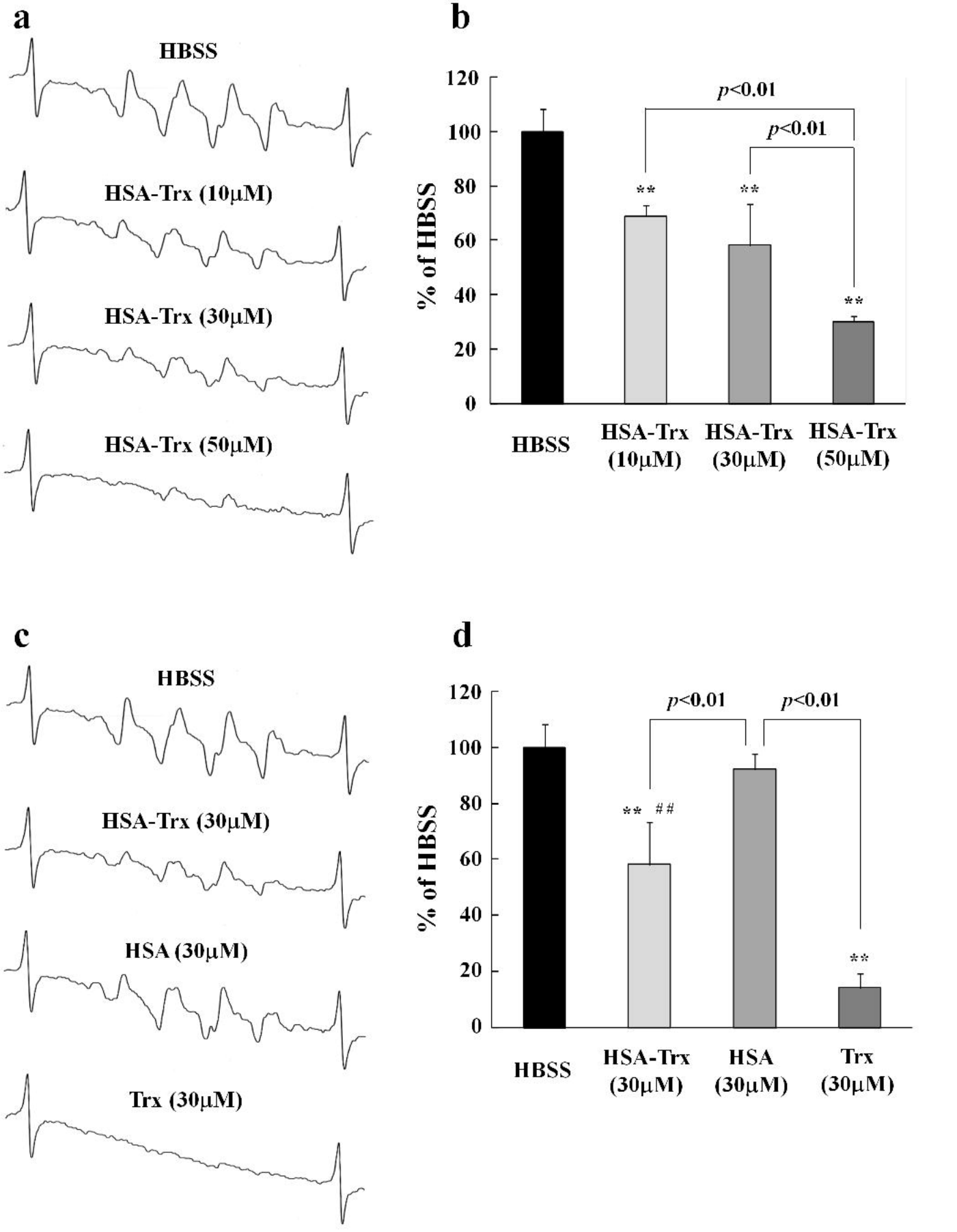

a
p<0.05
p<0.05
IL-6 levels (pg / whole lung)
300
250
200
150
100
50
0
**
*
BLM (-) Day 3 Day 3 Day 7 Day 7
BLM (+)
b
p<0.01
p<0.05
TNF-α levels (pg / whole lung)
120
100
80
60
40
20
0
**
**
BLM (-) Day 3 Day 3 Day 7 Day 7
BLM (+)
c
BLM (+)
BLM (-)
Saline (i.v.)
HSA-Trx (i.v.)
Day 3
MIF
β-actin
Day 7
MIF
β-actin
d
p<0.05
p<0.05
MIF / β-actin (% of BLM (-))
350
300
250
200
150
100
50
0
**
*
BLM (-) Day 3 Day 3 Day 7 Day 7
BLM (+)
Saline (i.v.)
HSA-Trx (i.v.)

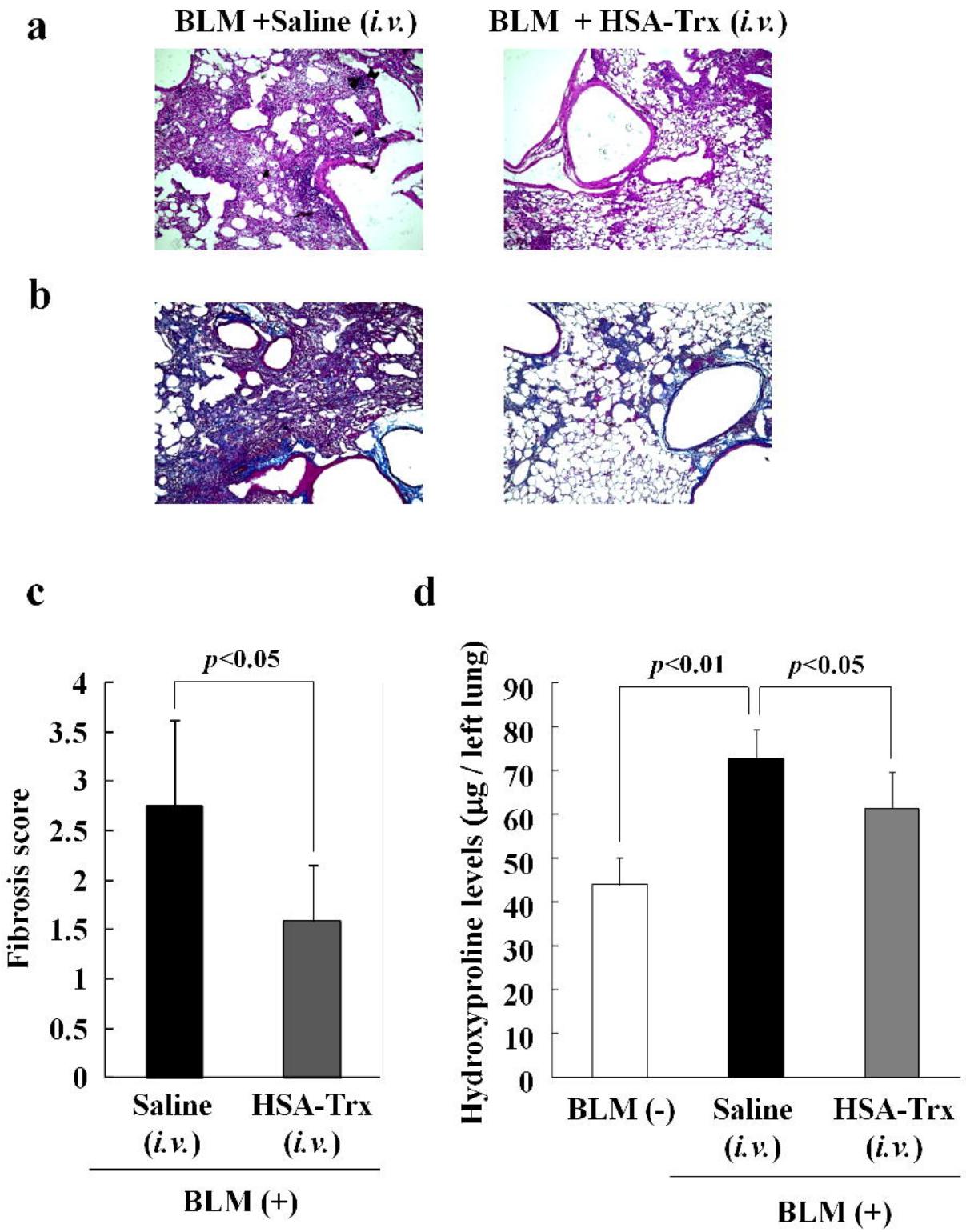

a
BLM (-)
BLM + Saline (i.v.)
BLM + HSA-Trx (i.v.)
8-OH-dG
b
NO2-Tyr

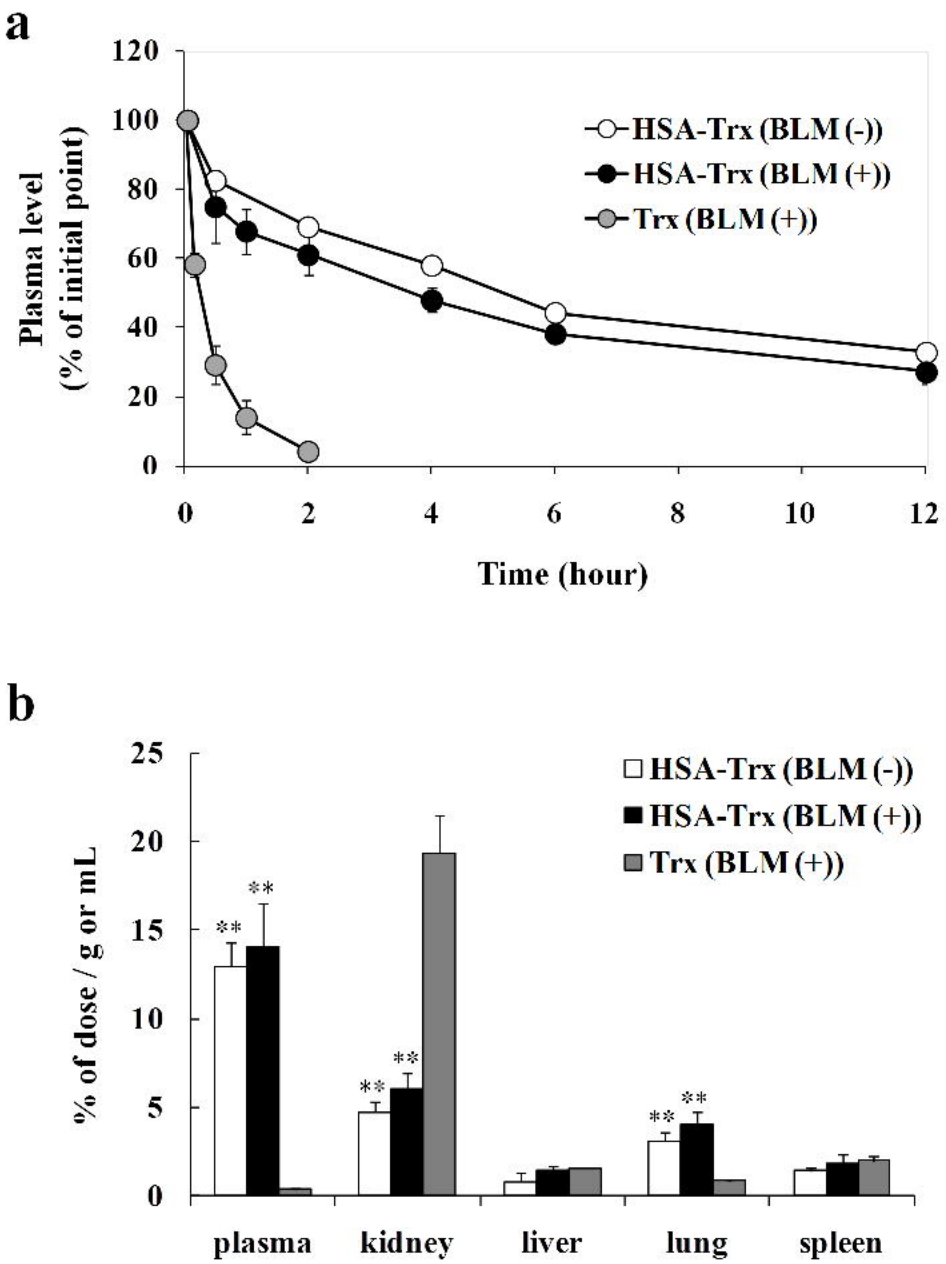

c
8-OH-dG-positive cells relative fluorescence (arbitrary units)
400
300
200
100
0
p<0.01
p<0.01
BLM (-)
Saline (i.v.)
HSA-Trx (i.v.)
BLM (+)


d
NO2-Tyr-positive cells relative fluorescence (arbitrary units)
250
200
150
100
50
0
p<0.05
p<0.05
BLM (-)
Saline (i.v.)
HSA-Trx (i.v.)
BLM (+)


e
MDA levels (nmol / left lung)
16
12
8
4
0
p<0.01
p<0.01
BLM (-)
Saline (i.v.)
HSA-Trx (i.v.)
BLM (+)


a
HBSS
HSA-Trx (10μM)
HSA-Trx (30μM)
HSA-Trx (50μM)
b
% of HBSS
120
100
80
60
40
20
0
p<0.01
p<0.01
**
**
**
HBSS
HSA-Trx (10μM)
HSA-Trx (30μM)
HSA-Trx (50μM)
c
HBSS
HSA-Trx (30μM)
HSA (30μM)
Trx (30μM)
d
% of HBSS
120
100
80
60
40
20
0
p<0.01
p<0.01
** ##
**
HBSS
HSA-Trx (30μM)
HSA (30μM)
Trx (30μM)


a
BLM +Saline (i.v.)
BLM + HSA-Trx (i.v.)
b
c
p<0.05
Fibrosis score
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline (i.v.)
HSA-Trx (i.v.)
BLM (+)
d
p<0.01
p<0.05
Hydroxyproline levels (μg / left lung)
90
80
70
60
50
40
30
20
10
0
BLM (-)
Saline (i.v.)
HSA-Trx (i.v.)
BLM (+)


a
Plasma level
(% of initial point)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Time (hour)
HSA-Trx (BLM (-))
HSA-Trx (BLM (+))
Trx (BLM (+))
b
% of dose / g or mL
25
20
15
10
5
0
**
plasma
kidney
liver
lung
spleen
HSA-Trx (BLM (-))
HSA-Trx (BLM (+))
Trx (BLM (+))