Long-Acting Human Serum Albumin-Thioredoxin Fusion Protein Suppresses Bleomycin-Induced Pulmonary Fibrosis Progression

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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 (O2•−). There is currently no effective treatment of 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 antioxidative 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 were 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-transforming growth factor (TGF)-β levels in the lung and inhibited the increase of inflammatory cells in bronchoalveolar lavage fluid, pulmonary inflammatory cytokines, and oxidative stress markers. An in vitro EPR experiment using phosphate-buffered saline–stimulated neutrophils confirmed the O2•− 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, because of its long-acting antioxidative 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% because of 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 recovery 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 levels of reactive oxygen species (ROS), such as superoxide anion radical (O2•−), and a decrease in the levels of glutathione and superoxide dismutase (SOD) in blood and bronchoalveolar lavage fluid (BALF) in patients with IPF have been reported (Beek et al., 2002; Psathakis et al., 2006). In addition, the genetic knockout of NADPH-oxidase, which increases the pulmonary level of O2•−, 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 and 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 interleukin (IL)-6, tumor necrosis factor (TNF)-α, and migration inhibitory factor (MIF), are markedly increased in the lungs of BLM-induced disease model animals, and it has also been reported that the knockout of IL-6 or the administration of anti-TNF-α or MIF antibodies suppresses BLM-induced lung disorders (Piguet et al., 1989, 1993; Tanino et al., 2002; Chaudhary et al., 2006; Saito et al., 2008). Therefore, drugs with both antioxidative and anti-inflammatory properties would be expected to be useful in the treatment of IPF.

Thioredoxin-1 (Trx) is a small redox-active protein (Mr 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 antioxidative effect, which is derived from dithiol-disulfide exchange in its active site, Trx also has anti-inflammatory properties, mainly because of its ability to inhibit neutrophil chemotaxis to inflammatory sites and to suppress the expression and activation of the macrophage MIF (Nakamura et al., 2001; Tamaki et al., 2006). Because of its desirable antioxidative and anti-inflammatory properties, Trx represents a new and potentially effective therapeutic agent for the treatment of IPF. However, because Trx is eliminated extensively via glomerular filtration, its plasma half-life is only approximately 1 hour in mice and 2 hours in rats, which is extremely short in terms of producing a significant therapeutic impact (Nakamura et al., 2001; Ueda et al., 2006). 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. (2003) demonstrated that exogenous recombinant Trx was effective in inhibiting BLM-induced lung damage 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) with use of a Pichia expression system. The plasma half-life of the HSA-Trx fusion protein in normal mice was found to be similar to that of HSA, which is 10 times longer than the plasma half-life of Trx (Ikuta et al., 2010). Of interest, HSA-Trx showed a higher modulation effects.

through its long-acting antioxidative and anti-inflammatory

Materials and Methods

BLM was purchased from Nippon Kayaku (Tokyo, Japan). Mayer’s hematoxylin, a 1% eosin alcohol solution, mounting medium for histologic 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 phosphor 12-nitroso trans 13-acetate (PMA) were obtained from Sigma-Aldrich (Tokyo, Japan). Paraformaldehyde, trichloroacetic acid (TCA), per-chloric acid, and 4-dimethylamino benzaldehyde were from Nacalai Tesque (Kyoto, Japan). Diethylene-triamine-pentaacetic acid was purchased from Dajindo Laboratories (Kumamoto, Japan); 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was purchased from Alexis Biochemicals (Lausen, Switzerland). The TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems Inc. (Minneapolis, MN). IL-6 and TNF-α ELISA kit were purchased from Biolegend (San Diego, CA). Sea-ICR mice (age, 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 in accordance with 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 liters of growth phase media, BMGY (1% yeast extract, 2% peptone, 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 days (OD680 = 2). It was then cultured in 800 ml of protein induction phase media, BMMY (1% yeast extract, 2% peptone, 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 days at 30°C. Methanol was added every 24 hours so that the concentration of methanol was maintained at 1% to sustain the protein expression induction effect. The secreted fusion protein was isolated from the growth media as follows. 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) at the medium was dialyzed against the same buffer. The column was washed with ~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 3 M NaCl. Next, with use of AKTA prime, the eluate was loaded onto a column of 5 ml Hitrap Phenyl HP column (GE Healthcare) for hydrophobic chromatography with the following conditions (buffer A, 0 mM Tris-HCl/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 (1967). The fusion protein was analyzed using 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 guide lines 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 phosphate-buffered saline (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/mouse) via the mouse tail vein 30 minutes before BLM treatment (day 0). HSA-Trx was administered intraperitoneally on days 0 and 7 (refer to every 1 week, Fig. 1A) or only on 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 was administered on day 7 after the BLM treatment. (Fig. 1C).
Histopathological Analysis of Lung Tissue (Hematoxylin and Eosin 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 hematoxylin and eosin (H&E) 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) phosphomolybdic acid], 0.75% (wt/vol) Orange G solution, solution C [0.12% (wt/vol) xyline 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. H&E and Masson’s trichrome staining samples were mounted with malinol and inspected using a microscope (BZ-8000; Keyence, 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%). Only fields in which 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, 1981). 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 hours at 110°C. Each sample was incubated for 20 minutes at room temperature after the pellets were hydrolyzed in 0.5 ml of 10 N HCl for 16 hours at 110°C. The supernatants were collected by centrifugation at 4100 g for 5 minutes at 4°C to separate the cells in the BALF from the liquid. Cells were dissolved in 0.9% NaCl, and the lysate was centrifuged again. From the recovered cells, the total cell lysate was centrifuged (4°C, 1000 g, 10 minutes). The recovered supernatants were collected by cannulating the trachea and lavaging the lung with 1 ml of sterile PBS containing 50 U/ml heparin (two times). Approximately 1.8 ml of BALF was routinely recovered from each animal. The BALF was centrifuged at 4100g for 5 minutes at 4°C to separate the cells in the BALF from the liquid. Cells were dissolved in 0.9% NaCl, and the 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 4°C for 2 hours 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 hours. The recovered lungs were covered with OCT compound and frozen at –80°C. Next, the lungs frozen with cryostat (CM3000i; Leica, Wetzlar, Germany) 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 solubilize the lung slice, followed by blocking with Block Ace (Dainippon Pharmaceuticals, Osaka, Japan) at room temperature for 15 minutes. Next, the primary antibody reaction was conducted below 4°C overnight. In addition, the primary mouse monoclonal antibody against 8-hydroxy-2'-deoxygenase (8-OH-dG) [15A3] (sc-66036; Santa Cruz Biotechnology Inc.), which has cross-reactivity with mouse, or the primary rabbit polyclonal antibody against nitrotyrosine (NO2-Tyr;AB5411; Millipore, Billerica, MA), which has cross-reactivity with mouse, was diluted to 2 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 hours. For the secondary antibody, in relation to NO2-Tyr and 8-OH-dG, Alexa Fluor 546 goat anti-mouse IgG (H+L; Invitrogen) and Alexa Fluor 488 goat anti-mouse IgG (H+L; Invitrogen) diluted 200 times were respectively used. After the reaction, the slide was observed using a microscope (BZ-8000; Keyence). Image analyses of the extent and intensity of 8-OH-dG and NO2-Tyr staining were also performed using ImageJ software.

**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 minutes of gravity sedimentation, the upper layer, containing leukocytes, was removed and centrifuged at 620 g for 10 minutes. The cell pellet was resuspended in 0.9% NaCl and underlaid with Ficoll-Paque (GE Healthcare). After centrifugation for 30 minutes at 1490 g, the mononuclear cell layer was isolated, and contaminating red blood cells were removed by hypotonic lysis. After centrifugation for 10 minutes 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 ROS.** The scavenging activity of HSA-Trx against O_{2-} released from neutrophils was determined using EPR spin trapping with DMPO. The neutrophils (1.0 × 10^6 cells/ml) were pretreated with PMA (1 μg/ml) for 7 minutes at 37°C to activate the cells and generate ROS. Aliquots of this cell suspension were combined with 100 μM DTPA in Hanks’ balanced salt solution 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 minutes of 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 minutes; field modulation width, 0.25 mT; receiver gain, 630; and time count, 0.3 seconds.

Radiolabeling of Proteins with $^{125}$I. HSA-Trx and Trx were radiolabeled with $^{125}$I according to the procedures reported previously (Watanabe et al., 2001; Furukawa et al., 2011) and purified using a Pharmacia Bio-Gel PD-10 column. The radiolabeled proteins were diluted with nonlabeled 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 (~10$^5$ cpm/mouse). Approximately 500 µl of blood was collected from the vena cava at 0.05, 0.167, 0.5, 1, 2, 4, 6, and 12 hours 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 gamma-counter (ARC-5000; Hitachi Aloka Medical, Tokyo, Japan).

Data Analysis. Noncompartment model was used in the pharmacokinetic analyses after $^{125}$I proteins administration. Each parameter was calculated using the program MOMENT (BS) (Tabata et al., 1996). Data are means ± S.D. for the indicated number of animals.

Significant differences among each group were examined using a one-way of analysis of variance followed by Tukey’s 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). Because Hoshino et al. (2003) found that the multiple administration of Trx (3.5 nmol/body) prevented BLM-induced pulmonary fibrosis, we adopted a dose of HSA-Trx equivalent to one Trx treatment (3.5 nmol/body).

Figure 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 using H&E staining (Fig. 2A), Masson’s trichrome staining (Fig. 2B), the quantity analysis of the section positively stained for collagen and displaying alveolar wall thickening (D), Hydroxyproline levels in lung tissue (Fig. 2D) on day 14. H&E staining showed that BLM administration induced severe lung damage (thickened and edematous alveolar walls

![Fig. 3. Effects of HSA-Trx (intravenously; every 1 week), Trx (intravenously; every 1 week), or Trx (intraperitoneally; every 2 days) on the BLM-induced pulmonary fibrosis. (A and B) Sections of pulmonary tissue were prepared 14 days after BLM administration and subjected to histopathological examination ([A] H&E 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 days after BLM administration. Each bar represents the mean ± S.D. (C, n = 4; D, n = 5). **P < 0.01; or *P < 0.05 versus BLM(+), saline (i.v.).](https://www.aspetjournals.org/journals/jpet)
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. 2, B–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. 2, A–D). Therefore, HSA-Trx was administered once per week in the subsequent experiments.

**Effect of HSA-Trx on Histologic Alterations and Hydroxyproline Levels in Lung Tissue.** Hoshino et al. (2003) demonstrated that, when Trx is administered every 2 days, it prevents BLM-induced pulmonary fibrosis. Thus, we compared the effect of the intravenous administration of HSA-Trx at weekly intervals with that for the intravenous administration of Trx at weekly intervals or the intraperitoneal administration of Trx at 2-day intervals (Fig. 1B).

H&E 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 indicated that HSA-Trx, when administered at 1-week intervals, significantly suppressed the lung injury and fibrosis in diseased model mice. Intraperitoneal administration of Trx at 2-day intervals resulted in similar therapeutic effects as those for the HSA-Trx treatment at weekly intervals, but intravenous administration of Trx at weekly 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 (Kinnula et al., 2005; Chaudhary et al., 2006). 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 (i.v.)]. 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 (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. 6, C 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 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 NO2-Tyr, oxidized product of nucleic acids and proteins, respectively, and also quantification of lipoperoxidation final reaction substances, MDA, in lung sections were performed on day 3 after BLM administration. As shown in Fig. 7, the accumulation of 8-OH-dG, NO2-Tyr, and MDA in lung tissue increased in BLM-mice [BLM(+)], saline (i.v.)], compared with normal mice [BLM(−)], whereas HSA-Trx clearly suppressed the levels of these oxidative stress markers in the lungs.

To confirm whether HSA-Trx shows scavenging activity against O2− generated from neutrophils, we conducted ex vivo EPR spectroscopy with use of a DMPO spin-trapping technique. As shown in Fig. 8, although PMA-stimulated neutrophils generated O2− and increased EPR signaling, HSA-Trx significantly decreased the EPR signaling in a concentration-dependent manner (Fig. 8, A and B). We also compared the O2− scavenging activity among 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, whereas HSA alone did not significantly change the intensity of the signal (Fig. 8, C and D).

**Effect of Post-Treatment of HSA-Trx on BLM-Induced Pulmonary Fibrosis.** For future clinical applications, the postadministration effect of HSA-Trx against BLM-induced fibrosis was examined (Fig. 1C). Because HSA-Trx suppressed ROS production by neutrophils (Fig. 8), the effect of the postadministration 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 H&E 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 performed using BLM mice. As shown in Fig. 10, no significant difference between the plasma concentration-time profiles for HSA-Trx in BLM(+) mice and that in BLM(-) mice were found. The half-life of HSA-Trx was approximately 8 hours in both BLM (8.45 ± 0.85 hours) and normal (7.89 ± 0.23 hours) mice, which is more than 10 times longer than that of Trx (0.50 ± 0.02 hours) 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, in which 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 ovalbumin-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 serum and lungs of patients with IPF, compared with 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 antioxidative 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 patients with IPF. In this study, we evaluated the therapeutic effects of HSA-Trx on mice with BLM-induced pulmonary fibrosis and obtained four important findings. First, HSA-Trx suppressed the progression of pulmonary fibrosis when given once weekly. Second, the mechanism by which HSA-Trx inhibits lung damage induced by BLM can be attributed to the long-acting antioxidative and anti-inflammatory modulation effects of Trx. Third, the postadministration of HSA-Trx was effective in suppressing lung damage. Fourth, 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 did other conventional pathologic models, it is possible that the pathologic 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 because of its short half-life, whereas the once weekly 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

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**Fig. 6.** Effect of HSA-Trx on pulmonary inflammatory cytokines and chemokine in BLM mice. IL-6 levels (A) and TNF-α levels (C) in lung were determined, and (C and D) MIF expression in lung were confirmed and analyzed 3 and 7 days after BLM administration. Each bar represents the mean ± S.D. (A and B, n = 5–6; D, n = 3–4). **P < 0.01; or *P < 0.05 versus BLM(-).**

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The image shows bar charts depicting the levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in the lungs of BLM-induced pulmonary fibrosis mice treated with HSA-Trx. The graphs illustrate the suppression of these inflammatory cytokines by HSA-Trx treatment compared to control groups. Additionally, the expression of macrophage-inflammatory protein (MIF) is shown to be modulated by HSA-Trx administration.
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 pathologic 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 attributable to the antioxidative activity of Trx, because Trx exhibits superior antioxidative 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, NO2-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 O2⁻ 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 because of their dramatic increases at the beginning of inflammation (Fig. 5C) and subsequent release of a large amount of O₂⁻ (Manoury et al., 2005; Kinder et al., 2008). A comparison of the neutrophil-produced O₂⁻ scavenging capacities of HSA-Trx and Trx showed that HSA-Trx retained ~50% of the specific activity of Trx (Fig. 8). This reduced activity of HSA-Trx is likely to be 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) (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; Bellocq et al., 1999). Because HSA-Trx scavenges ROS, it should be able to suppress the production of active TGF-β1 in lung tissue. The findings reported here indicate that HSA-Trx significantly reduced the active TGF-β1 content in lung tissue induced by the BLM treatment (Fig. 4). Therefore, 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 turn, activates NADPH oxidase in fibroblasts, thereby enhancing ROS production and downregulating the expression of glutamate cysteine ligase (Thannickal and Fanburg, 1995; Arsalane et al., 1997), 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 to suppress BLM-induced pulmonary fibrosis.
Because 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 days of BLM treatment may inhibit this vicious circle.

In addition to its antioxidative action, Trx has anti-inflammatory characteristics, including its ability to suppress the migration and/or 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 decreased the lung concentrations of these cytokines in mice with BLM-induced lung disorders (Fig. 6, A and B). Although 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 (Tamaki et al., 2006; Son et al., 2009). MIF promotes the production of TNF-α, IL-6, and other cytokines, as well as ROS, such as nitric oxide and superoxide anions (Baugh and Bucala, 2002). MIF is expressed at increased levels in a broad range of oxidative stress situations and inflammation-related pathologic 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). It is noteworthy that HSA-Trx was also able to significantly suppress MIF expression induced by BLM administration (Fig. 6, C and D). Taking all these findings into consideration, the inactivation of MIF by HSA-Trx may also involve the anti-inflammatory effects of HSA-Trx observed in this study.

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 postadministration 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 antioxidative and anti-inflammatory modulator. In recent years, acute exacerbation of IPF due to conditions, such as a cold or influenza, along with its progression, have contributed to the mortality among patients with IPF, 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.

**Fig. 9.** Effects of postadministration of HSA-Trx (every 1 week) against BLM-induced pulmonary fibrosis 1 day after BLM treatment. (A and B) Sections of pulmonary tissue were prepared 14 days after BLM administration and subjected to histopathological examination ([A] H&E 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 days after BLM administration. Each bar represents the mean ± S.D. (C, n = 4; D, n = 5).
Although 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 patients with IFP with a possibility of fortnightly or monthly administrations, considering the fact that the half-life of HSA in humans is approximately 20 days (Peters, 1985). Although further investigations related to establishing the treatment regimen need to be performed, a reduced administration frequency and stable therapeutic effect of HSA-Trx would make the treatment more convenient, safe, and cost-effective and improve the health-related quality of life for patients with IFP. The BLM animal model is widely used in the assessment of potential antifibrotic agents. However, the aspect of slow and irreversible progression of IFP 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 the clinical setting as opposed to preventing the response evoked by BLM.

Authorship Contributions

**Participated in research design:** R. Tanaka, Watanabe, Kodama, Chuang, Ishima, Hamasaki, K. Tanaka, Mizushima, Otagiri, Maruyama.

**Conducted experiments:** R. Tanaka, Watanabe, Kodama, Ishima.

**Contributed new reagents or analytic tools:** R. Tanaka, Watanabe, Kodama.

**Performed data analysis:** R. Tanaka, Watanabe, Kodama.

**Wrote or contributed to writing of the manuscript:** R. Tanaka, Watanabe, Chuang, Otagiri, Maruyama.

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**References**


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