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Recombinant human thioredoxin-1 becomes oxidized in circulation and suppresses bleomycin-induced neutrophil recruitment in the rat airway

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Abstract
Thioredoxin-1 (TRX) is a redox-active protein with anti-inflammatory effects. This study investigated the optimal delivery method and the mechanisms of recombinant human TRX (rhTRX) to suppress neutrophil recruitment in a rat bleomycin (BLM)-induced sustained acute lung injury model. In male Wister rats intratracheally administered with 0.125 mg/kg BLM, 8 mg/kg/day rhTRX was intravenously administered on days 3–6 using one of three protocols: daily bolus injection, 3 h daily infusion or continuous infusion for 96 h. Only the continuous-infusion of rhTRX significantly reduced the neutrophil infiltration compared with the other two methods. The BLM-induced down-regulation of L-selectin expression on circulating neutrophils was inhibited by rhTRX. Oxidized rhTRX showed a comparable effect with reduced rhTRX and rhTRX incubated with plasma or circulating in plasma was more than 99% oxidized. These results suggest that rhTRX becomes oxidized in circulation and continuous infusion of rhTRX suppresses neutrophil recruitment in the airway.

Keywords: *Airway inflammation, neutrophil, oxidative stress, redox status, thioredoxin*

Introduction

Human thioredoxin-1 (TRX) was originally purified from the supernatant of human T-cell leukaemia virus type-I-transformed T-cells and identified as an adult T-cell leukaemia-derived factor [1]. TRX is secreted by cells in response to oxidative stresses, such as virus infection, ultraviolet irradiation and hydrogen peroxide [2,3]. Elevated plasma/serum levels of TRX are good indicators of systemic oxidative stress and are evident in various disorders including human immunodeficiency virus-1 (HIV-1) and hepatitis C infections, autoimmune diseases, ischemia–reperfusion, chronic heart failure and acute respiratory distress syndrome (ARDS) [4–9].

When the concentration of circulating recombinant human TRX (rhTRX) administered in the blood exceeds the intracellular level of endogenous TRX, it has an anti-chemotactic effect against neutrophil extravasation into the inflammatory site [10]. This is because leukocytes migrate to the stronger chemotactic factor, which is the rhTRX in the blood [11].

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As the concentration of TRX in leukocytes and tissues is 100 ~ 1000 ng/ml [3,12], an rhTRX blood concentration > 1 μg/ml is necessary for an anti-chemotactic effect. This suppresses neutrophil recruitment and attenuates inflammatory disorders such as cerebral infarction, acute pancreatitis and autoimmune myocarditis and arthritis [13–15].

Acute lung injury (ALI) or ARDS is caused by neutrophil extravasation into the pulmonary interstitial space and the alveolar space associated with pulmonary oedema [16]. In spite of artificial lung support and other conventional therapy [17], the prognosis of ALI/ARDS remains poor and new therapeutic strategies are keenly anticipated. The administration of rhTRX has been shown to suppress lung injuries caused by ischemia–reperfusion and inflammatory cytokines [18,19]. Accordingly, we are conducting a translational research programme to clinically apply rhTRX to ALI/ARDS patients. Recently, we reported that rhTRX administration suppresses lipopolysaccharide (LPS)-induced bronchoalveolar neutrophil infiltration in the rat [20]. However, the optimal dose and mode of delivery to humans remain to be clarified. In the current study, we established a sustained lung injury rat model by intratracheal instillation of bleomycin (BLM) and evaluated the protective effects of different rhTRX therapeutic protocols. BLM is frequently used to produce experimental lung fibrosis [21]; however, it was used in the current study to induce sustained neutrophil infiltration into the rat airway. Moreover, we investigated the mechanisms how circulating rhTRX suppresses neutrophil infiltration in the airway. Circulating rhTRX becomes more than 99% oxidized in plasma and suppresses BLM-induced down-regulation of L-selectin on circulating neutrophils.

Materials and methods

Animals

Pathogen-free male Wistar rats weighing 220–240 g were obtained from Nihon SLC (Hamamatsu, Japan). The animal study protocol was approved by the Animal Research Committee of the Graduate School of Medicine of Kyoto University, Japan.

Reagents

BLM was obtained from Nihon Kayaku, Tokyo, Japan. Sodium *N*-(2-[4-{2, 2-dimethylpropionyloxy} phenylsulphonyl-aminobenzoyl] amino-acetate tetrahydrate) (sivelestat) was obtained from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Recombinant hTRX was obtained from Redox Bio Science, Inc. (Kyoto, Japan) (originally from Ajinomoto, Co. Inc., Kawasaki, Japan). The redox status of rhTRX was measured by the 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) assay, as previously described [7]. Reduced rhTRX was lyophilized and stored at −80°C. Half-oxidized rhTRX was prepared by incubating lyophilized rhTRX at 50°C for 1 week. Fully-oxidized rhTRX was prepared by the addition of hydrogen peroxide. The numbers of SH residues per molecule were 5.0, 2.6 and 0.0 for the reduced, half-oxidized and fully-oxidized rhTRX, respectively.

Experimental design

Under anaesthesia, the rats were intratracheally instillated with 0.125 mg/kg BLM. On days 3–6, the animals were intravenously injected with rhTRX in saline according to one of three protocols: bolus injection once daily, 3 h infusion once daily or 96 h continuous infusion. On day 7, bronchoalveolar lavage (BAL) was performed 10 times with 5 ml Hanks' balanced salt solution. All cells were collected, counted and centrifuged on slide-glasses by the Cytospin system (Thermo Electron Corporation, Waltham, MA). In total, 500 cells were counted under light microscopy and the percentage of neutrophils was calculated. In some animals, the lungs were fixed with buffered 10% formalin that was intratracheally administered with 20 cm H_2O pressure on day 7.

Flow cytometry

L-selectin (CD62L) expression on circulating neutrophils was analysed by adding 5 μl PE-conjugated hamster anti-rat CD62L antibody (BD Pharmingen, San Jose, CA) to 100 μl blood taken on day 7 of the experiment and then incubating at 4°C for 30 min. After adding lysis buffer, the sample was centrifuged by 400 g for 5 min and resuspended in FACS buffer. L-selectin (CD62L) expression was analysed by FACS Calibur (BD Biosciences, Mountain View, CA) using Flowjo software (Tree Star, Ashland, OR).

Immunohistochemistry

Neutrophil detection was performed using an anti-myeloperoxidase (MPO) antibody (Neo Markers, Fermont, CA). Paraffin-embedded 6-mm-thick sections were dewaxed and antigen retrieval was performed by heating in a microwave in 10 mm citrate buffer (pH 6.0) for 10 min. After biotin blocking (Dako Biotin Blocking X0590, Dako, Kyoto, Japan) and pre-incubation in 2% bovine albumin in phosphate-buffered saline (PBS), the slides were incubated with anti-MPO rabbit polyclonal antibody as a primary antibody or normal rabbit Immunoglobulin G (IgG; Upstate, Charlottesville, VA) as a negative control at room temperature for 1 h. Biotin-conjugated anti-rabbit IgG (AK-5001, Vector Laboratories, UK) was used as a secondary antibody and the reaction was visualized with the avidin–biotin

complex (Vectastain ABC-AP AK-5000, Vector Laboratories) followed by the chromogen (Vectastain alkaline phosphatase substrate kit II, SK-5100, Vector Laboratories). Counterstaining was performed with haematoxylin.

Redox blotting

Human TRX contains five cysteines per molecule (C32, C35, C62, C69 and C73). When it is half-oxidized, one disulphide bond is formed in the active site (C32–C35). After further oxidation, a second disulphide bond is formed between C62 and C69 [22]; a dimer or multimer might subsequently be formed between the C73 of two or more molecules. Redox blotting analysis was performed as described previously, with some modifications, to detect the redox status of rhTRX [22]. Briefly, rhTRX was carboxymethylated in guanidine-Tris solution (6 m guanidine–HCl, 50 mm Tris [pH 8.3], 3 mm EDTA and 0.5% [v/v] Triton X-100) containing 50 mm iodoacetic acid (IAA). After incubation at 37°C for 30 min, excess IAA was removed by Sephadex chromatography (Microspin G-25 column, Amersham Biosciences, Amersham, UK). Eluates were diluted in 5 × sample buffer (0.1 mm Tris-HCl [pH 6.8], 50% [v/v] glycerol and 0.05% [w/v] bromophenol blue) and separated on a discontinuous native polyacrylamide gel (5% stacking, 15% resolving gel). Proteins were detected by Coomassie blue staining.

Western blotting

Western blotting using an anti-human TRX antibody was performed as previously described, with some modifications [23]. Proteins were separated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and then electrically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with 5% skimmed milk in Tris-based saline containing 0.05% Tween-20 (T-TBS) at room temperature for 1 h. The membrane was then incubated with an anti-human TRX mouse monoclonal antibody (ADF-11, Redox Bioscience, Inc.) in T-TBS containing 5% skimmed milk at 4°C overnight. After incubation with horseradish peroxidase-conjugated anti-mouse IgG antibody, detection by chemiluminescence was performed with ECL™ Western blotting detection reagents (Amersham Biosciences).

Redox-Western blotting

Redox western blotting was performed as described previously, with some modifications [22]. After redox blotting (as described above), the proteins were transferred to a PVDF membrane and were detected by an anti-human TRX antibody according to the western blotting method.

Statistical analysis

Statistical differences were determined using the Student's t-test at a significance level of $p < 0.05$.

Results

Intratracheal instillation of BLM-induced neutrophil recruitment in the rat airway

To examine the time-dependent effect of BLM, the total number of neutrophils and the percentage of neutrophils in the BAL fluids were examined on days 1, 2, 4, 6, 9 and 14 after intratracheal instillation of 1 mg/kg BLM. As shown in Table I, neutrophil recruitment in the airway was maximal on day 2 and continued for >9 days after BLM instillation. On examining the dose effect of BLM on day 1, we observed that 0.125 mg/kg was sufficient to cause neutrophil recruitment (Table II). Based on the data shown in Table III, this dosage was chosen for intratracheal instillation to produce a sustained ALI rat model, which could be used to evaluate the therapeutic effect of rhTRX on neutrophil recruitment in the airway.

Continuous infusion of rhTRX suppressed bronchoalveolar neutrophil infiltration

Of the three different administration protocols, only 96 h of continuous rhTRX infusion was effective in suppressing the number of neutrophils and the percentage of neutrophils in the BAL fluids (Figure 1A and B). Immunohistochemistry using an anti-MPO antibody showed that 0.125 mg/kg BLM induced slight neutrophil infiltration into the alveolar space, whereas continuous infusion of 8 mg/kg/day rhTRX inhibited the infiltration (Figure 1C). Western blotting using anti-TRX polyclonal antibody

Table I. Time course of total number of neutrophils and percentage of neutrophils in BAL fluids after intratracheal instillation of 1 mg/kg BLM.

Day	1	2	4	6	9	14
Total neutrophils ($\times 10^6$/rat)	1.79 ± 0.30	4.69 ± 0.97	2.94 ± 0.59	1.75 ± 0.35	0.75 ± 0.17	0.24 ± 0.07
Percentage of neutrophils	53.2 ± 8.75	68.5 ± 7.78	62.0 ± 4.39	42.3 ± 10.3	19.4 ± 0.71	6.58 ± 2.28

$n = 4$ for each group. All data are presented as mean ± SD.

Table II. Effects of BLM concentration on total number of neutrophils and percentage of neutrophils in BAL fluids 1 day after intratracheal instillation.

Bleomycin	1	0.5	0.25	0.125	0.0625	0
Total neutrophils ($\times 10^6$/rat)	1.79	1.73	1.94	0.90	0.82	0.08
Percentage of neutrophils	53.2	49.1	48.4	29.2	24.2	1.2

$n = 2$–4 for each group. All data are presented as the mean.

(rabbit serum) which cross-reacts with human and rat TRX [24] showed that plasma levels of endogenous rat thioredoxin was less than 100 ng/ml even after bleomycin treatment (Figure 1D). We previously reported that plasma levels of exogenous administered rhTRX were more than 1 μg/ml in rat plasma by 8 mg/kg/day infusion, suggesting that more than 10-fold higher amount of TRX is necessary for the inhibition of bronchoalveolar neutrophil infiltration.

Comparable effects of 8 mg/kg/day and 16 mg/kg/day rhTRX doses

To evaluate the optimal rhTRX dose, 4 mg/kg/day, 8 mg/kg/day and 16 mg/kg/day were applied via 96 h continuous infusion and the corresponding rat rhTRX blood levels were found to be 777 ± 173 ng/ml, 1783 ± 271 ng/ml and 3347 ± 403 ng/ml, respectively. The 16 mg/kg/day, but not the 4 mg/kg/day, dosage had a comparable effect to that of 8 mg/kg/day in suppressing the percentage of neutrophils in BAL fluids (Figure 2A).

Sivelestat had no effect on the suppression of bronchoalveolar neutrophil infiltration

The neutrophil elastase inhibitor sivelestat is used at a dosage of 4.8 mg/kg/day as a treatment for ALI/ARDS. In the LPS-induced ALI rat model, sivelestat suppressed bronchoalveolar neutrophil infiltration as well as rhTRX (data not shown). By contrast, continuous infusion of sivelestat (8 mg/kg/day) did not have a suppressive effect on neutrophil recruitment in the airway in the BLM-induced sustained ALI model (Figure 2B).

rhTRX suppressed BLM-induced down-regulation of L-selectin (CD62L) on circulating neutrophils

To address the mechanism of the anti-chemotactic effect of rhTRX, L-selectin (CD62L) expression on circulating neutrophils was analysed using a flow-cytometer. Previously, we reported that the intravenous injection of rhTRX suppressed LPS-induced activation of p38 mitogen activating kinase (MAPK) in neutrophils and the down-regulation of L-selectin expression on extravasated neutrophils [10]. Here, we found that 96 h of continuous infusion of 8 mg/kg/day rhTRX blocked the BLM-induced downregulation of L-selectin on circulating neutrophils (Figure 3).

Half-oxidized rhTRX showed a comparable suppressive effect to reduced rhTRX

The redox status of rhTRX was demonstrated by redox western blotting analysis (Figure 4A). Reduced rhTRX contained five reduced SH residues, half-oxidized rhTRX mainly contained one disulphide bond and fully-oxidized rhTRX existed as multimers. Interestingly, half-oxidized rhTRX, but not fully-oxidized rhTRX, showed a comparable effect to that of reduced rhTRX in suppressing neutrophil recruitment (Figure 4B). Interestingly, when rhTRX was incubated with rat plasma *in vitro* at room temperature for 10 min, its redox status shifted to oxidized (Figure 4C). Western blotting showed that rhTRX incubated with rat plasma *in vitro* at 37°C for 30 min was almost in a dimer form, which was similar to half-oxidized rhTRX (Figure 4D).

Circulating rhTRX were oxidized

To confirm the redox status of circulating rhTRX in plasma *in vivo*, plasma samples were collected from the rats 24 h after continuous intravenous infusion of 150, 300 and 600 mg/kg/day rhTRX and analysed by western blotting and redox western blotting. In plasma *in vivo*, circulating rhTRX was mainly in a monomer form, although there were some dimers and heterodimers with larger proteins in plasma. Redox western blotting showed that it contained mainly only one reduced cysteine residue, suggesting that circulating rhTRX was mainly an oxidized form

Table III. Time course of total number of neutrophils and percentage of neutrophils in BAL fluids after intratracheal instillation of 0.125 mg/kg BLM.

Day	1	3	7	10
Total neutrophils ($\times 10^6$/rat)	0.90 ± 0.05	0.58 ± 0.22	0.58 ± 0.11	0.34 ± 0.05
Percentage of neutrophils	29.2 ± 5.2	24.9 ± 6.5	25.3 ± 3.7	12.5 ± 2.3

$n = 3$–5 for each group. All data are presented as the mean.

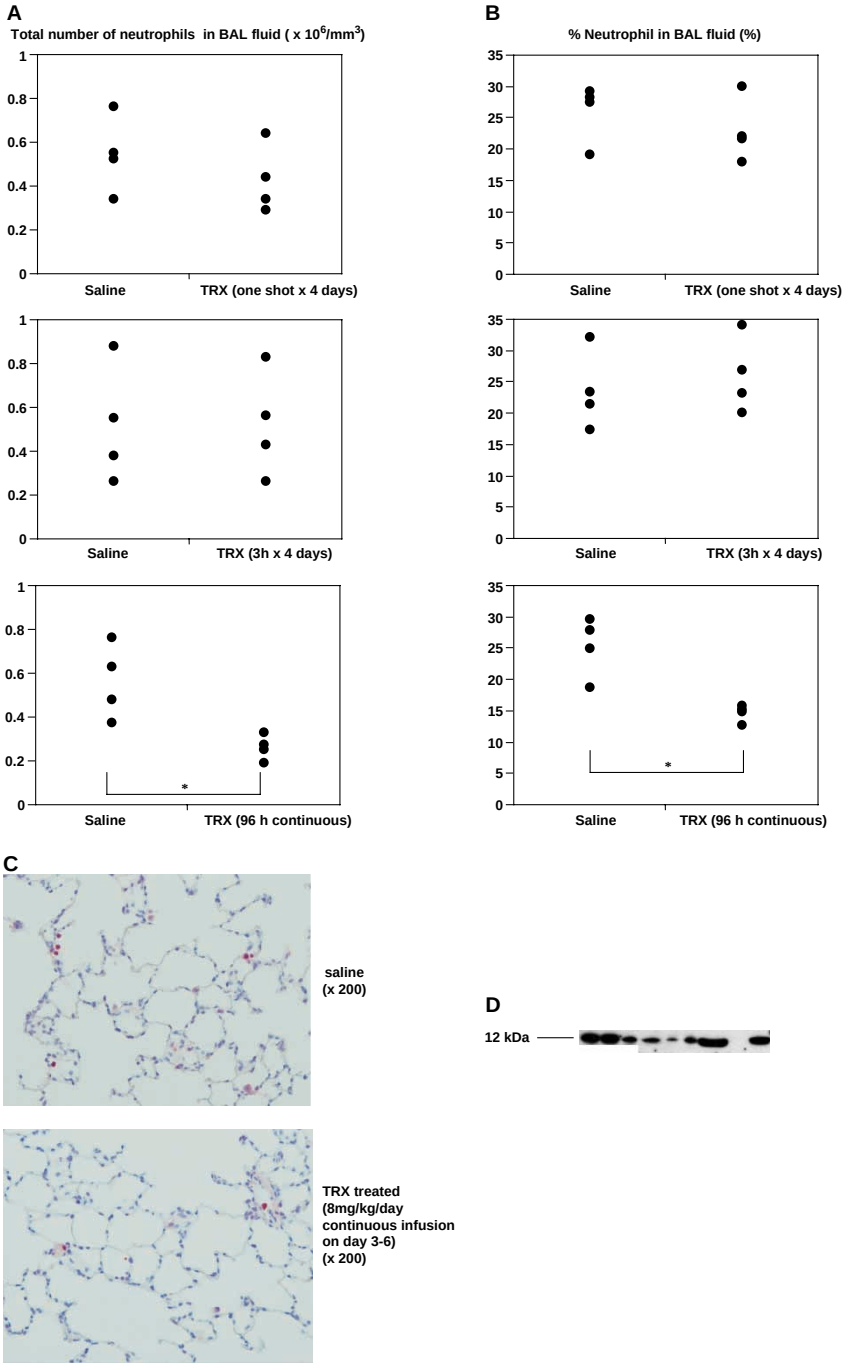

Figure 1. (A) Total number of neutrophils in BAL fluid ($\times 10^6$/mm^3). Bolus injection, 3 h infusion and continuous 96 h infusion of rhTRX on days 3–6 were compared with control saline ($n = 4$ for each group). Asterisk indicates the statistical significance (*$p < 0.05$). A significant difference ($p = 0.0158$) was observed between the continuous infusion and control groups. (B) Percentage of neutrophils in the BAL fluid. Bolus injection, 3 h infusion and continuous 96 h infusion of rhTRX on days 3–6 were compared with control saline ($n = 4$ for each group). A significant difference ($p = 0.0056$) was observed between the continuous infusion and control groups. (C) Anti-MPO staining of the lungs 7 days after intratracheal instillation of 0.125 mg/kg BLM. The saline-treated lung is shown in the upper panel ($\times 40$) with higher magnification ($\times 100$). The TRX-treated lung is shown in the lower panel ($\times 40$). (D) Western blotting using anti-TRX polyclonal antibody (rabbit serum) which cross-reacts with human and rat TRX: lane 1, 2, plasma from rat received 0.125 mg/kg BLM it + 8 mg/kg/day rhTRX iv; lane 3, 4, plasma from rat received 0.125 mg/kg BLM it; lane 5,6, normal rat plasma; lane 7, rat red blood cell lysate; lane 8, rhTRX 10 ng/ml; lane 9, rhTRX 100 ng/ml. Samples were separated by SDS-PAGE under reducing condition. This antibody cross-reacted with rat red blood cell lysate and rhTRX.

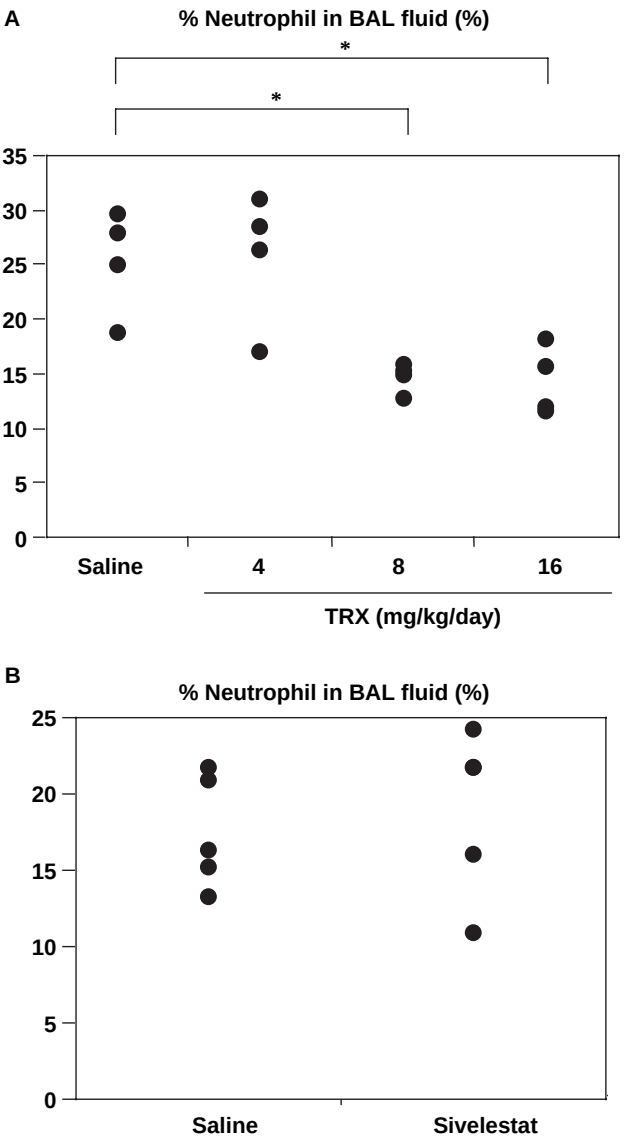

Figure 2. Percentage of neutrophils in BAL fluid. (A) The percentages of neutrophils were significantly lower in the 8 mg/kg/day ($p = 0.0056$) and 16 mg/kg/day ($p = 0.0088$) rhTRX groups. (B) There was no significant difference between saline and sivelestat (8 mg/kg/day) treatment ($n = 4$ for each group).

containing two intramolecular disulphides and one free cysteine (Figure 5).

Discussion

We previously reported that intraperitoneal injection of rhTRX suppressed BLM-induced and inflammatory cytokine-induced sub-acute interstitial pneumonia [19]. Prior to commencing a translational research project for the treatment of ALI/ARDS patients with intravenous infusion of rhTRX, we have prepared the recombinant protein in compliance with good manufacturing practice. However, the optimal dose and method of delivery have not yet been clarified.

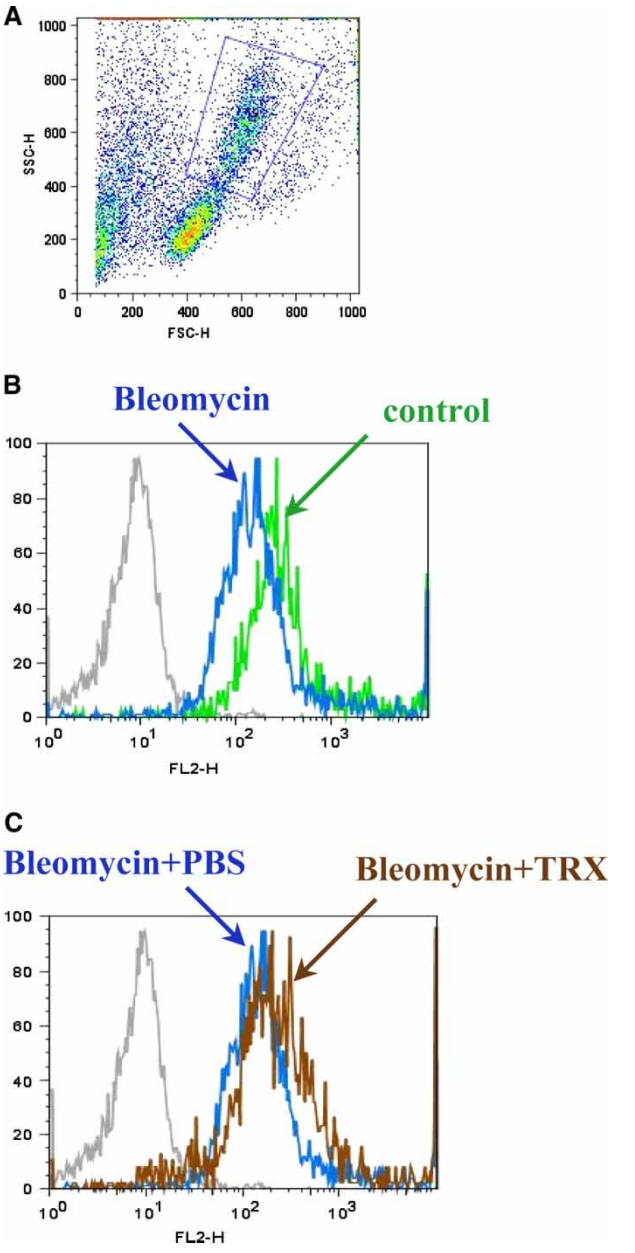

Figure 3. L-selectin (CD62L) expression on circulating neutrophils. (A) Neutrophils from whole-blood leukocytes were gated and analysed. (B) Intratracheal instillation of BLM down-regulated L-selectin (CD62L) expression on circulating neutrophils. (C) 96 h continuous infusion of 8 mg/kg/day rhTRX inhibited BLM-induced down-regulation of CD62L expression on circulating neutrophils.

The present results suggest that the most effective rhTRX dose is 8 mg/kg/day in this rat model and that the optimal method of administration is continuous infusion. Previous work showed that a continuous infusion of 8 mg/kg/day rhTRX maintained a blood rhTRX concentration of >1 μg/ml in the rat [20]. Our present data support this finding. This concentration has been previously shown to significantly suppress neutrophil extravasation into an inflammatory site in mice [10]. Our unpublished preliminary

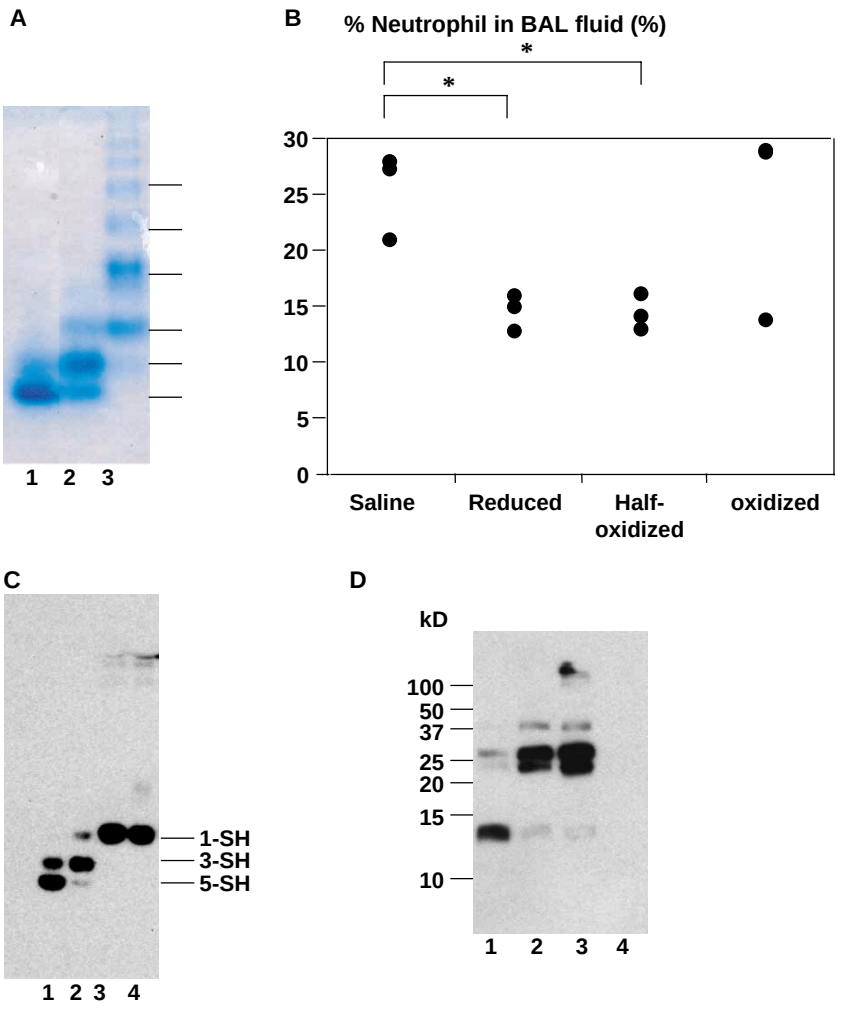

Figure 4. (A) Redox blotting of rhTRX: lane 1, reduced rhTRX; lane 2, half-oxidized rhTRX; and lane 3, fully-oxidized rhTRX. The lowest band represents the reduced form of the molecule with five cysteines, the second lowest band represents one disulphide bond and three cysteines per molecule and the third lowest band represents two disulphide bonds (C32–C35 and C62–C69) and one cysteine (C73) per molecule. The upper bands represent multimers. (B) Percentage of neutrophils in BAL fluid. Reduced TRX and half-oxidized TRX showed significantly lower percentages of neutrophils ($n = 3$ for each group). Asterisk indicates the statistical significance ($*p < 0.05$). (C) Redox western blotting analysis using anti-human TRX antibody after redox blotting of rhTRX: lane 1, reduced rhTRX; lane 2, half-oxidized rhTRX; lane 3, fully-reduced rhTRX incubated with rat plasma at room temperature for 10 min; and lane 4, half-oxidized rhTRX incubated with rat plasma at room temperature for 10 min. The lowest band represents the fully reduced form and the second lowest band represents the half-oxidized form containing one disulphide bond per molecule. The third lowest band represents two disulphide bonds per molecule. (D) Western blotting analysis of rhTRX: lane 1, reduced rhTRX; lane 2, half-oxidized rhTRX; lane 3, reduced rhTRX incubated with rat plasma at 37°C for 30 min; and lane 4, rat plasma. Samples were separated by SDS-PAGE under non-reducing condition.

data showed that a continuous infusion of rhTRX at 2 mg/kg/day was sufficient to maintain a blood concentration of > 1 μg/ml in the monkey, suggesting that ≤ 2 mg/kg/day might be enough to maintain a blood concentration of > 1 μg/ml in humans. The optimum administrative dose for humans will be investigated by a dose-determining protocol.

The anti-inflammatory effects of TRX are not completely understood. Recently, the inflammatory cytokine macrophage migration inhibitory factor (MIF) has been reported to have a redox-active motif (-Cys-Xxx-Xxx-Cys-) and to be a member of the TRX family. Moreover, TRX and MIF regulate one another's expression reciprocally [25]. The interaction of TRX and MIF in the regulation of inflammation is currently under investigation.

Previously, we reported that intravenous injection of rhTRX suppressed the LPS-induced down-regulation of L-selectin (CD62L) on extravasated neutrophils in the mouse air-pouch model. The down-regulation of L-selectin on the cell surface of neutrophils was mediated by shedding and was a marker of activation to promote the extravasation of neutrophils [26]. In the current study, we found that the intratracheal instillation of BLM induced the down-regulation of L-selectin on circulating neutrophils and that this was inhibited by rhTRX administration, thereby supporting the mechanism by which rhTRX

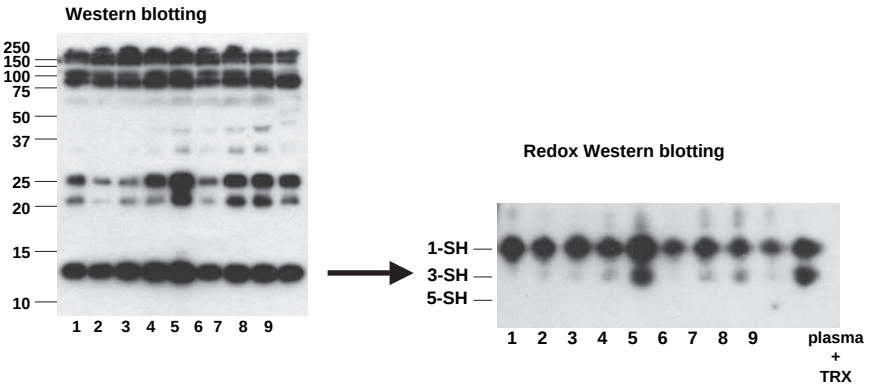

Figure 5. Left panel: Western blotting using anti-human TRX antibody for plasma samples from rats plasma 24 h after continuous intravenous infusion of 150 (lanes 1–3), 300 (lanes 4–6) and 600 (lanes 7–9) mg/kg/day rhTRX (reduced as administered). Samples were separated by SDS-PAGE under non-reducing conditions. Circulating rhTRX was mainly in a monomer form *in vivo*, although there were some dimers and heterodimers with other proteins. Right panel: Redox western blotting showed that circulating monomer rhTRX mainly contained only one reduced cysteine residue.

suppresses neutrophil recruitment into the airway. This also partly explains the anti-inflammatory effects of TRX.

The present result showed that rhTRX almost completely becomes a dimer when it is incubated with plasma *in vitro* and circulating rhTRX is oxidized *in vivo*, suggesting that circulating rhTRX in plasma is considered as a non-reductant. Moreover, oxidized rhTRX was shown to have a comparable effect to that of reduced rhTRX in the suppression of BLM-induced bronchoalveolar neutrophil infiltration. Interestingly, when rhTRX was incubated with plasma *in vitro*, it becomes dimer easily. In contrast, circulating rhTRX is an oxidized form but not as easily a dimer *in vivo.* It is possible that some reductant may suppress the dimer formation of rhTRX *in vivo*. Previous studies have shown that the antioxidant *N*-acetylcysteine did not suppress ALI induced by endotoxin [27]. We previously reported that oxidized rhTRX show the comparable effect to reduced rhTRX in the anti-chemotactic function on neutrophils in the mouse air-pouch model [10], while oxidized, but not reduced, TRX inhibits the secretion of the potent inflammatory cytokine interleukin (IL)-1β from macrophages [28]. Taken together, these results indicate that the anti-inflammatory effect of administered rhTRX is mainly due to anti-chemotaxis.

However, it is also possible that oxidized rhTRX might be reduced at the cell surface or after being taken up into cells to produce its effect. Previously, we reported that mutant rhTRX, in which two cysteines in the active site were replaced with serines, showed no anti-inflammatory effects [14]. It suggested that the reversible disulphide/dithiol exchange is necessary for the anti-inflammatory effect. In fact, we recently found that TRX is existing on the cell surface of endothelial cells [29]. Extracellular oxidized TRX might be reduced by membrane-bound reduced TRX at the cell surface.

The cellular uptake of circulating TRX is limited [20], although it is released from cells through a leaderless pathway. The reduction of oxidized TRX is mediated by NADPH and TRX reductase; however, it is not yet known whether the recycling of oxidized/reduced TRX is possible extracellularly, such as in the circulation of the blood. Therefore, the contribution of an anti-oxidative effect of TRX might be limited.

The neutrophil elastase inhibitor sivelestat has been reported to suppress ALI/ARDS in animal models [30,31]. A previous report has shown that TRX itself has anti-elastase activity [32]. We previously reported that rhTRX showed a suppressive effect in an LPS-induced ALI model [20], where sivelestat also showed the comparable effect as rhTRX (data not shown). However, in the present sustained ALI model caused by intratracheal BLM, rhTRX was more effective than sivelestat. A recent study revealed that sivelestat had no apparent efficacy in the treatment of ALI/ARDS patients [33]. TRX may have more potential as a new therapeutic strategy against ALI/ARDS than sivelestat, since it has potential anti-oxidative, anti-apoptotic and anti-inflammatory effects in addition to its anti-elastase activity. The clinical efficacy of rhTRX compared with that of sivelestat will be clarified in a future phase III clinical trial.

ARDS/ALI is often caused by anti-cancer agents such as gefitinib, which is a molecular-targeted agent that inhibits epidermal growth factor receptor (EGFR) tyrosine kinase. Although the intracellular expression of TRX in cancer tissues is associated with resistance to anti-cancer agents [34,35], there is no evidence that exogenously administered rhTRX promotes the growth of tumours in nude mice [20]. In addition, administered rhTRX did not inhibit anti-cancer agents used to suppress the growth of the same tumour [20]. This might be because the cellular uptake of exogenous TRX is limited and any

administered TRX that comes into contact with the plasma immediately becomes oxidized, leading to a loss of tumour growth activity.

In conclusion, we determined that the optimal mode of rhTRX delivery was continuous infusion and 8 mg/kg/day was the most effective dose to maintain blood concentrations of >1 μg/ml in a rat model. Future work will establish protocols for ALI/ARDS patient clinical trials involving the continuous infusion of rhTRX.

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