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ORIGINAL ARTICLE

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Endogenous and Exogenous Thioredoxin 1 Prevents Goblet Cell Hyperplasia in a Chronic Antigen Exposure Asthma Model

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ABSTRACT

Background: Goblet cell hyperplasia with mucus hypersecretion contribute to increased morbidity and mortality in bronchial asthma. We have reported that thioredoxin 1 (TRX1), a redox (reduction/oxidation)-active protein acting as a strong antioxidant, inhibits pulmonary eosinophilic inflammation and production of chemokines and Th2 cytokines in the lungs, thus decreasing airway hyperresponsiveness (AHR) and airway remodeling in mouse asthma models. In the present study, we investigated whether endogenous or exogenous TRX1 inhibits goblet cell hyperplasia in a mouse asthma model involving chronic exposure to antigen.

Methods: We used wild-type Balb/c mice and Balb/c background human TRX1-transgenic mice constitutively overproducing human TRX1 protein in the lungs. Mice were sensitized 7 times (days 0 to 12) and then challenged 9 times with ovalbumin (OVA) (days 19 to 45). Every second day from days 18 to 44 (14 times) or days 35 to 45 (6 times), Balb/c mice were treated with 40 μg recombinant human TRX1 (rhTRX1) protein. Goblet cells in the lungs were examined quantitatively on day 34 or 45.

Results: Goblet cell hyperplasia was significantly prevented in TRX1-transgenic mice in comparison with TRX1 transgene-negative mice. rhTRX1 administration during OVA challenge (days 18 to 44) significantly inhibited goblet cell hyperplasia in OVA-sensitized and -challenged wild-type mice. Moreover, rhTRX1 administration after the establishment of goblet cell hyperplasia (days 35 to 45) also significantly ameliorated goblet cell hyperplasia in OVA-sensitized and -challenged wild-type mice.

Conclusions: Our results suggest that TRX1 prevents the development of goblet cell hyperplasia, and also ameliorates established goblet cell hyperplasia.

KEY WORDS

airway remodeling, asthma, goblet cell, thioredoxin

INTRODUCTION

Bronchial asthma is a chronic inflammatory disease of the lower airways, characterized clinically by reversible airway obstruction and airway hyperresponsiveness (AHR). The characteristic feature of asthma is airway inflammation including infiltration by inflammatory cells such as eosinophils and lymphocytes, epithelial damage, and airway remodeling.[1,2] Airway remodeling is observed in both children and adults with asthma, and is characterized pathologically by goblet cell hyperplasia, airway wall edema, and hyperplasia and hypertrophy of smooth muscle cells and myofibroblasts.[3,4] The structural changes in

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the airways are suggested to be responsible for thickening of airway walls, airway flow limitation, and airway hyperresponsiveness (AHR) in patients with severe asthma.[5]

Goblet cell hyperplasia in the airway is an important pathologic characteristic of bronchial asthma, regardless of disease severity, because previous studies have shown that goblet cell hyperplasia with mucus hypersecretion contributes to increased morbidity and mortality.[6-8] Indeed, the airways of patients who die of severe acute asthma may have more than a 30-fold increase of goblet cell hyperplasia compared with patients who die of non-acute asthma.[6] Therefore, mucus hypersecretion by hyperplastic goblet cells in the airways contributes to the pathogenesis of asthma.[9] In addition, goblet cell hyperplasia with mucus hypersecretion is also observed in the lungs of asthma model animals.[10,11]

Thioredoxin (TRX) is a redox (reduction/oxidation)-active protein. Two thioredoxins are cytosolic (TRX1) and mitochondrial (TRX2).[12] TRX1 plays a role in the protein disulfide/dithiol reducing system with thioredoxin reductase and NADPH. Reduced TRX1 reduces oxidized protein.[13] Therefore, TRX1 is thought to act as a strong scavenger for reactive oxygen species (ROS) and as an antioxidant both *in vitro* and *in vivo*.[13-18] We previously showed that TRX1 inhibited AHR and recruitment of eosinophils into the lungs in mouse models of asthma.[19] Recently, we reported that TRX1 prevents the development of airway remodeling, eosinophilic pulmonary inflammation, and AHR in a chronic antigen exposure asthma model. Administration of recombinant human TRX1 (rhTRX1) protein also improves established airway remodeling in this asthma model.[20]

In the present study, we used Balb/c background human TRX1-transgenic (Tg) mice, in which human TRX1 protein was constitutively overproduced in the lungs. We investigated whether endogenous or exogenous TRX1 was able to inhibit goblet cell hyperplasia in a murine model of chronic asthma induced by exposure to ovalbumin (OVA). We also examined whether rhTRX1 administration after the establishment of airway remodeling ameliorated goblet cell hyperplasia in this mouse asthma model.

METHODS

MICE AND REAGENTS

Wild-type Balb/c mice were purchased from Charles River Japan (Yokohama, Japan). Recombinant human TRX1 protein (rhTRX1) was purchased from Oriental Yeast Co., Ltd. (Shiga, Japan). rhTRX1 administration did not induce any lethality or pulmonary inflammation in mice. TRX1 treatment did not increase the activity of myeloperoxidase in the lungs of mice, as reported previously.[20]

GENERATION OF BALB/c BACKGROUND TRANSGENIC MICE CONSTITUTIVELY OVERPRODUCING HUMAN TRX1

We previously established human TRX1 transgenic mice that constitutively overproduce human TRX1 protein in the lungs using the human elongation factor (EF)-1α promoter.[18] In this study, Balb/c human TRX1 transgenic mice were generated by mating more than 5 times with wild-type Balb/c mice, as reported previously.[20]

STUDY DESIGN

The experimental protocol is shown in Figure 1. Seven- to 10-week-old female Balb/c TRX1-Tg positive (+), TRX1-Tg negative (–) littermate mice, or wild-type Balb/c mice were treated seven times with an intraperitoneal injection of 10 μg sterile chicken OVA (grade V, Sigma-Aldrich Chemical, St. Louis, MO, USA) emulsified with 4 mg of sterile aluminum hydroxide (Alu-Gel-S Suspension, Serva Electrophoresis GmbH, Heidelberg, Germany) in a total volume of 200 μL. The injections were given every 2 days from days 0 to 12. These mice, each housed in a closed box, were challenged for 48 minutes with 0.9% saline (vehicle) (group A) or 5% OVA in 0.9% saline (group B), administered via the airways by an ultrasonic nebulizer (Omron NE-U07, Tokyo, Japan), amounting to a total of 1, 3, 6, 9, or 12 challenges. Mice in groups C and D were sensitized with OVA and then challenged for 48 minutes with 5% OVA in 0.9% saline as described above. Every second day from days 18 to 44 (14 times in group C) or days 35 to 45 (six times in group D), mice were treated with an intraperitoneal injection of 40 μg rhTRX1 suspended in 100 μL of sterile phosphate-buffered saline (PBS). On day 20, 25, 34, 46, or 54 (24 hours after the last OVA aerosol), the mice were euthanatized and pulmonary histopathologic analysis was performed.

HISTOLOGICAL EXAMINATIONS

The histological analysis was performed as reported previously.[16-20] Briefly, mice were euthanatized by an intraperitoneal injection of pentobarbital sodium (2.5 to 5 mg per mouse). After the thorax had been opened, the lungs were fixed immediately by intratracheal instillation of 20% buffered formalin for 15 to 20 minutes at a constant pressure of 27 cmH_2O. After gross examination, the extracted tissues were placed in 20% buffered formalin and further fixed for at least 24 hours. Sections (4 μm thick) were cut from paraffin-embedded tissues and placed on poly-l-lysine coated slides and then incubated overnight at 55 to 60℃. Deparaffinized sections were stained with hematoxylin and eosin (HE) and alcian blue-periodic acid-Schiff (AB-PAS).

MORPHOMETRIC ANALYSIS

Serial paraffin sections of the right and left lungs of

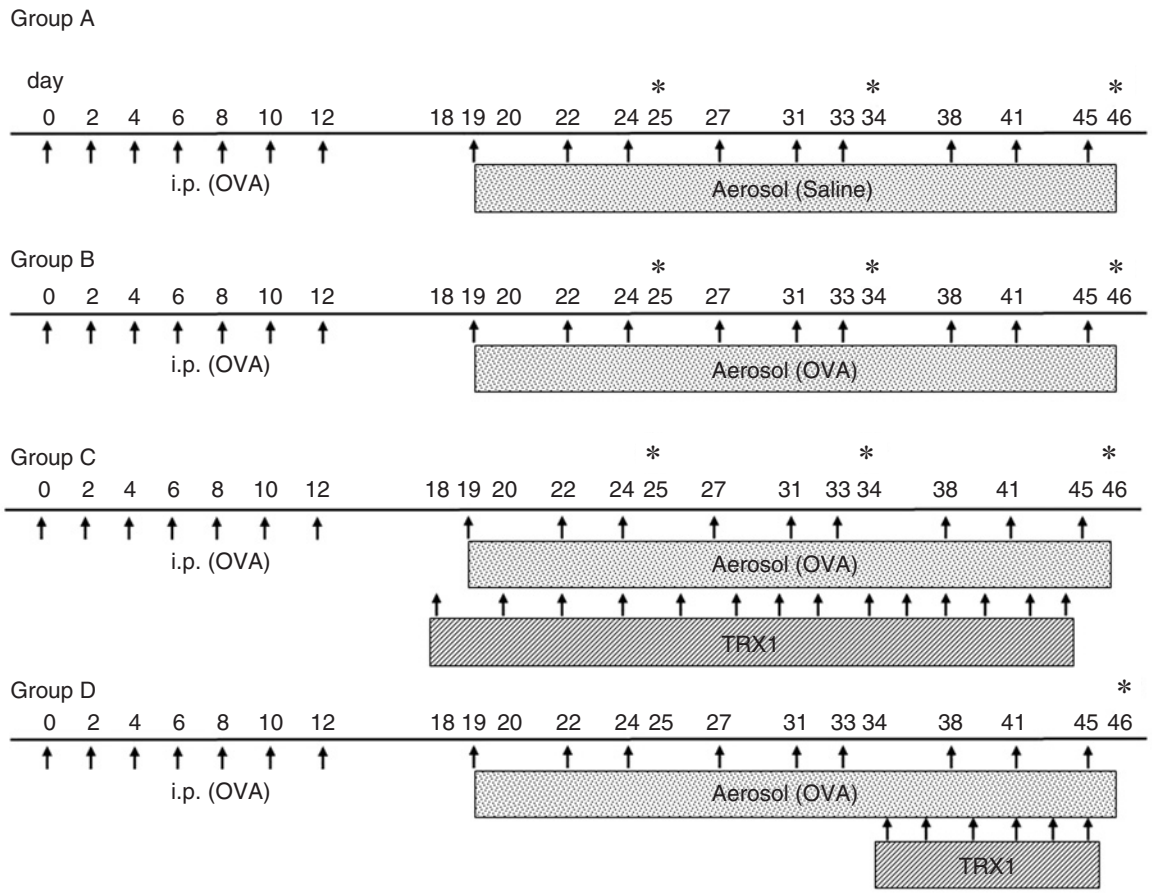

Fig. 1 Schematic summary of the experimental protocol. Group A: Mice were sensitized with 10 μg of ovalbumin (OVA) plus 4 mg of Al $(OH)_3$ on days 0, 2, 4, 6, 8, 10, and 12, and then challenged by inhalation of 0.9% saline on days 19, 22, 24, 27, 31, 33, 38, 41, and/or 45. Group B: Mice were sensitized with OVA, and then challenged with 5% OVA (w/v) in 0.9% saline on days 19, 22, 24, 27, 31, 33, 38, 41, and/or 45. Group C: Mice were sensitized, and then challenged with OVA, as described for group B. The mice were then treated intraperitoneally with 40 μg recombinant human TRX1 protein (rhTRX1) every 2 days from days 18 to 44. Group D: Mice were sensitized and then challenged with OVA, as described for group B. The mice were injected intraperitoneally with 40 μg rhTRX1 every 2 days from days 35 to 45. *Mice were sacrificed for histopathological examination on days 25, 34, and 46.

each mouse were made. Then, digitized video images of the entire lung fields were captured by a Nikon Microphot-FX microscope (Nikon, Tokyo, Japan) with a CCD camera (DXM1200, Nikon). The video output of the camera was sent to a microscopic-imaging workstation (Nikon ACT-1, Nikon). Goblet cells were defined as cells with large AB-PAS-stained areas (greater than or equal to one-third of the height of the epithelium from the basement membrane to the luminal surface), and cells with sparse and light staining were carefully excluded, as we have reported previously.[21] AB-PAS-positive goblet cells in the tracheal epithelium were hand-counted in 5 to 10 random high-power fields (hpfs) (observation at 400×) in a double-blind manner, as reported previously.[17,21] In the serial sections, the tissues were stained with HE, and the total number of epithelial cells was also counted in the tracheal epithelium.

STATISTICAL ANALYSIS

Results are expressed as means ± standard error of the mean. Comparisons among three or more groups were made by ANOVA. Differences were considered significant at $P < 0.05$. SAS 9.1.3 software, Japanese edition (SAS Institute, Cary, NC, USA), was used for statistical analysis.

RESULTS

ESTABLISHMENT OF GOBLET CELL HYPERPLASIA IN A CHRONIC ANTIGEN EXPOSURE ASTHMA MODEL

Female wild-type Balb/c mice were sensitized with OVA, then challenged with OVA (group B) or 0.9% saline (group A). We histologically analyzed the lung

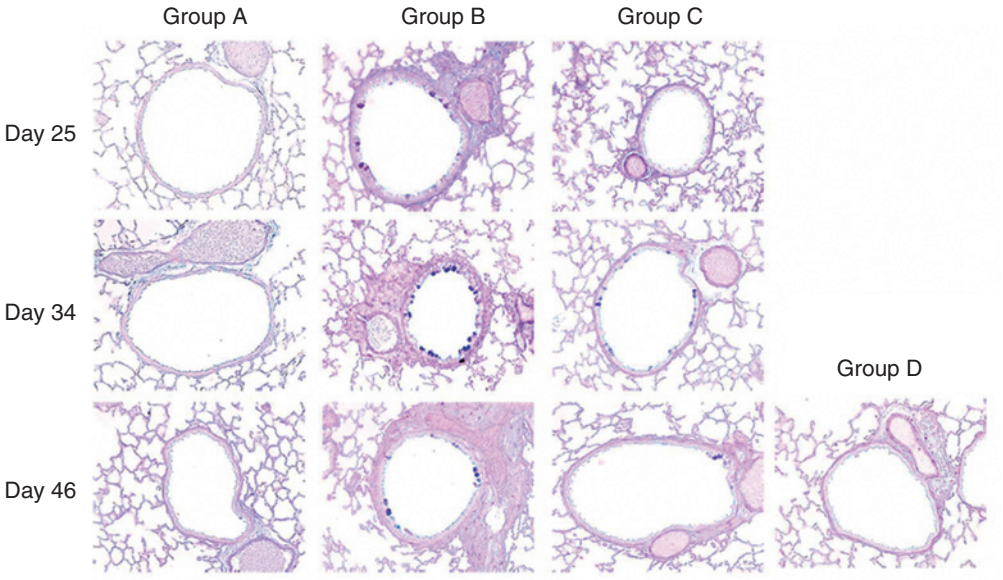

Fig. 2 Establishment of goblet cell hyperplasia in a chronic antigen exposure asthma model. The lung tissues were evaluated microscopically on days 25, 34, and 46 following alcian blue-periodic acid-Schiff (AB-PAS) staining. Original magnification×100.

tissues by AB-PAS staining on days 25, 34, and 46 (Fig. 2). Severe airway inflammation was found in OVA-sensitized/OVA-challenged mice (group B) but not in OVA-sensitized/0.9% saline-challenged mice (group A) after day 25. The smooth muscle layer of the airways was thickened in group B, but not in group A, on days 34 and 46, but not on day 25. On day 25, AB-PAS-positive goblet cells were observed in the tracheal epithelium in group B, but not in group A. On day 34, the maximum number of AB-PAS-positive goblet cells was observed; thus, goblet cell hyperplasia was established in the airways of group B. On day 46, goblet cells were decreased in comparison with day 34. Moreover, AB-PAS-positive goblet cells were barely evident in group B on day 54 (after 12 challenges with OVA) (data not shown). These findings demonstrated that airway remodeling accompanied by airway inflammation, smooth muscle hyperplasia, and goblet cell hyperplasia was established by day 34. Maximum hyperplasia of goblet cells was observed on day 34 in this chronic antigen exposure asthma model.

OVERPRODUCTION OF TRX1 IN LUNGS PREVENTS GOBLET CELL HYPERPLASIA

The promoter of human EF-1α is well known to have strong promoter activity in various types of cells.[22] A previous study reported that the use of human EF-1α promoter genes resulted in persistent and strong gene expression in mouse lung.[23] We previously established human TRX1 transgenic (Tg) mice under the control of the human EF-1α promoter, in which human TRX1 protein was overproduced in the lungs.[18] In this study, we investigated whether overproduction of human TRX1 protein was able to prevent hyperplasia of goblet cells in a mouse chronic OVA-exposure model. Female Balb/c TRX1 Tg (+) mice and TRX1 Tg (–) littermate mice were OVA-sensitized, then challenged with OVA (group B), or 0.9% saline (vehicle, group A). Mice were harvested on day 34. Our repeated experiments showed that smooth muscle hyperplasia and eosinophilic pulmonary inflammation was prevented in the OVA-sensitized and -challenged TRX1 Tg (+) mice, when compared with TRX1 Tg (–) mice, as we have reported previously[20] (data not shown). AB-PAS-positive goblet cells were greatly increased in the tracheal epithelium of TRX1 Tg (–) mice. In contrast, goblet cells were greatly decreased in the tracheal epithelium of TRX1 Tg (+) mice (Fig. 3A). Quantitative analysis revealed that AB-PAS-positive goblet cells in the tracheal epithelium were significantly ($p < 0.05$) decreased in TRX1 Tg (+) mice, when compared with control Tg (–) littermates (Fig. 3B). These results show that overproduction of TRX1 in the lungs prevents goblet cell hyperplasia in this chronic antigen exposure model.

EXOGENOUS TRX1 INHIBITS GOBLET CELL HYPERPLASIA

We performed independent experiments ($n = 6$) to examine whether rhTRX1 treatment during OVA challenge was able to inhibit goblet cell hyperplasia in this chronic OVA-exposure model. We performed quantitative analysis of goblet cell hyperplasia on days 20, 25, 34, and 46. The number of AB-PAS-

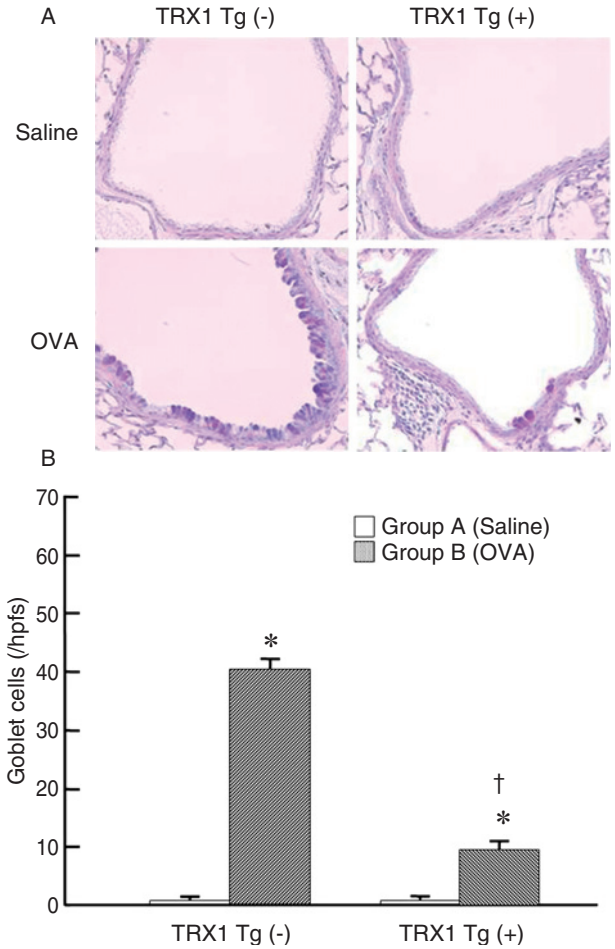

Fig. 3 Overproduction of TRX1 in the lungs prevents goblet cell hyperplasia in a chronic antigen exposure model. Female Balb/c-background TRX1 transgene (Tg) positive (+) and control TRX1-Tg negative (-) littermate mice were sensitized with OVA. Then, mice were challenged with 0.9% saline (Group A) or OVA (treated as Group B), as shown in Figure 1. (**A**) The lung tissues were evaluated microscopically on day 34 after AB-PAS staining. Original magnification ×400. (**B**) Quantitative analysis. AB-PAS-positive goblet cells in the tracheal epithelium were hand-counted in 5 to 10 random high-power fields (hpfs) (observation at ×400) in a double-blind manner as described in the Methods section. Goblet cells were counted as AB-PAS-positive cells per hpf. Numbers of Tg (-) mice in groups A and B were 5 and 7, respectively. Numbers of Tg (+) mice in groups A and B were 4 and 3, respectively. *$P < 0.05$ versus Group A, † $P < 0.05$ versus Group B.

positive goblet cells in the tracheal epithelium of OVA-sensitized/OVA-challenged mice (group B) was significantly ($P < 0.05$) greater than that in control mice (group A) on days 20, 25, 34 and 46 (Fig. 4). We had previously reported that the half-life of rhTRX1 is 51.3 hours in the mouse lung.[17] Therefore, 40 μg of rhTRX1 was administered intraperitoneally to OVA-sensitized and -challenged mice (group C). Histological analysis showed that goblet cell hyperplasia and pulmonary inflammation were significantly ($P < 0.05$) decreased in group C on days 20, 25, 34 and 46, when compared with group B. The number of goblet cells in group C was significantly ($P < 0.05$) lower than that in group B on days 20, 25, 34 and 46 (Fig. 4). These results showed that administration of hrTRX1 during antigen challenge was able to prevent goblet cell hyperplasia in this chronic antigen exposure asthma model.

TRX1 ADMINISTRATION AFTER ESTABLISHMENT OF AIRWAY REMODELING AMELIORATES GOBLET CELL HYPERPLASIA

Next, we examined whether rhTRX1 administration after establishment of airway remodeling would ameliorate goblet cell hyperplasia in this mouse asthma model induced by chronic exposure to OVA. As we previously reported, airway remodeling accompanied by eosinophilic pulmonary inflammation and smooth muscle hyperplasia was established by day 34 in this mouse asthma model,[20] and therefore we treated OVA-sensitized/OVA-challenged mice with 40 μg of rhTRX1 from days 35 to 45 every second day (group D in Fig. 1), and performed histological analysis on day 46. Goblet cell hyperplasia, as well as thickening of the smooth muscle layer in the airways and pulmonary inflammation, were markedly inhibited in group D on day 46, in comparison with group B (Fig. 2). Moreover, the number of goblet cells in group D was significantly ($P < 0.05$) lower than that in group B on day 46 (Fig. 4). These results show that exogenous TRX1 administration after the establishment of airway remodeling was able to improve goblet cell hyperplasia in this murine chronic OVA-exposure model.

DISCUSSION

Pulmonary inflammation, which is a characteristic of asthma, results in increased oxidative stress in the airways.[24,25] Pulmonary inflammatory cells (particularly eosinophils, macrophages, and neutrophils) release large amounts of ROS (reactive oxygen species) and RNS (reactive nitrogen species) in asthma. In fact, levels of the lysosomal enzymes myeloperoxidase (MPO) (from neutrophils and monocytes/macrophages) and eosinophil peroxidase (EPO) are increased in the peripheral blood, induced sputum, and bronchoalveolar lavage (BAL) fluid of patients with stable asthma.[24] These increased levels of MPO and EPO lead to the production of large amounts of hydroxyl radical (OH^-), which is a powerful ROS. This increased oxidative stress (ROS and RNS) may damage epithelial cells, and evoke AHR, which is characteristic of asthma.[24] Goblet cell hyperplasia is thought to be induced after epithelial damage by en-

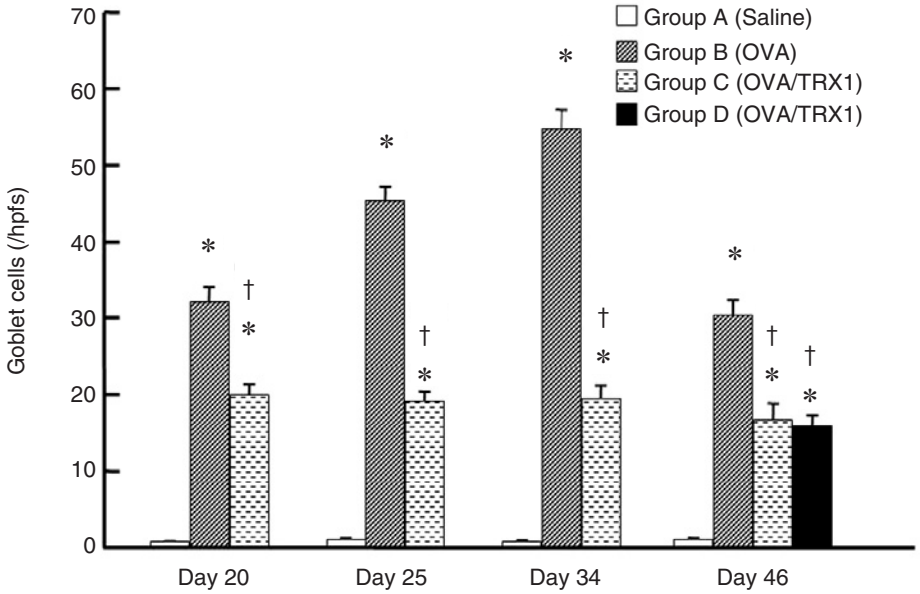

Fig. 4 Prevention of goblet cell hyperplasia by administration of recombinant human TRX1 protein. For quantitative analysis, AB-PAS-positive goblet cells in the tracheal epithelium were hand-counted in 5 to 10 random high-power fields (hpfs) (observation at ×400) in a double-blind manner. *$P < 0.05$ versus Group A, † $P < 0.05$ versus Group B ($n = 6$ per group at each time point).

dogenous ROS induced by various stimuli such as neutrophil elastase,[26] ozone,[27] endotoxin,[27] and diesel engine emissions.[30] ROS also directly stimulate histamine release from mast cells, thus increasing mucus secretion from airway epithelial cells in asthma.[28] In this study, we showed that both endogenous and exogenous TRX1 inhibited goblet cell hyperplasia in our mouse model of asthma induced by chronic exposure to OVA. Because TRX1 acts as a strong antioxidant, and reduces the oxidization of proteins or the level of H_2O_2, together with peroxiredoxin,[13,25] the protective effects of TRX1 against goblet cell hyperplasia in this mouse asthma model may be at least partly attributable to its antioxidant properties.

In patients with asthma, pulmonary inflammatory cells including CD4+ T cells, eosinophils, and neutrophils produce large amounts of chemokines and Th2 cytokines including eotaxin, macrophage inflammatory protein (MIP)-1α, RANTES (regulated on activation normal T cell expressed and secreted), IL-4, IL-5, and IL-13.[1,25] These chemokines and Th2 cytokines may play a role in airway flow limitation and AHR in asthma.[29] In addition, it has been demonstrated that the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 induce goblet cell hyperplasia in animal models and also *in vitro*.[30-33] A previous study showed that TRX1 directly suppresses the activation of neutrophils by attenuation of p38 MAPK activation induced by LPS and other chemokines.[34] It has been reported that TRX1 modulates cell signaling through ASK-1[35] and p53.[36] TRX1 also activates the function of some transcriptional factors such as NF-κB, p53, AP-1, Ref-1, and Sp-1.[13,25] We previously showed that TRX1 upregulates the expression of IL-1 family cytokines (IL-1α, IL-1β, IL-1 receptor antagonist [IL-1Ra], and IL-18) in a mouse asthma model.[19] Moreover, we recently found that TRX1 decreases the expression of chemokines and Th2 cytokines in the lung, including eotaxin, MIP-1α and IL-13, in Balb/c mice chronically exposed to antigen.[20] Therefore, TRX1 may inhibit goblet cell hyperplasia by inhibiting or activating certain signal pathways (such as ASK-1 and p38 MAP kinase) and transcription factors, the expression of cytokines and chemokines, and the chemotaxis of inflammatory cells, including neutrophils, in the lungs.

In this study, we did not perform recombinant TRX1 protein-dose dependent experiments to see whether recombinant TRX-1 can inhibit goblet cell hyperplasia in a dose-dependent manner. Therefore, we established human TRX1 transgenic mice constitutively overproducing human TRX1 protein in the lungs on a Balb/c background. Here we showed that human TRX1 Tg positive but not Tg negative mice can prevent goblet cell hyperplasia. These experiments may show that lung expression of TRX-1 protein can inhibit goblet cell hyperplasia in a dose-dependent manner. A previous study demonstrated that recombinant TRX1 inhibited CD4 coreceptor functions and decreased IL-2 production by CD4 wild-type T cells *in vitro*,[37] suggesting that TRX1 can pre-

vent antigen presenting cell (APC) funtion. However, we previously showed TRX1 did not modulate serum OVA-specific IgE levels in the mouse asthma model.[19] Our results suggest that TRX1 cannot inhibit APC function *in vivo*. Further analysis is needed to confirm this issue.

It has been shown that several genes are upregulated in association with goblet cell hyperplasia. Among them, MUC family genes encode various mucin glycoproteins that are primary secretory proteins (e.g., MUC-1, MUC-2, and MUC-4).[10] MUC5AC is the most predominant MUC gene expressed in the airways.[10] It has been demonstrated that the level of MUC5AC gene expression is increased in animal models of asthma and in tissue samples from asthmatic patients.[10,38] In addition, it has been reported that overexpression of the Gob-5 gene in airway epithelia exacerbates AHR, goblet cell hyperplasia, mucus overproduction, and eosinophil infiltration in a mouse asthma model.[39] Thus, TRX1 can prevent goblet cell hyperplasia by inhibiting the expression of MUC genes and/or the Gob-5 gene. Further analysis will be needed to verify this possibility.

The treatment strategy for asthma control consists mainly of the use of inhaled corticosteroids, because the pathophysiological abnormality of this disease is caused by pulmonary inflammation.[40] However, approximately 5% of patients do not respond to this therapy.[41] In addition, current therapeutic approaches, such as the use of corticosteroids to reverse established airway remodeling, have had very limited success in patients with severe asthma.[42,43] Therefore, effective therapies that are targeted at severe asthma are needed. We previously showed that exogenous TRX1 inhibits AHR and pulmonary inflammation in a mouse acute antigen exposure asthma model.[19] Furthermore, we recently demonstrated that exogenous TRX1 administration during the allergen challenge period inhibited smooth muscle hyperplasia and inflammation in the lungs of a chronic antigen exposure mouse asthma model.[20] Moreover, treatment with rhTRX1 after the airway structural changes had become established improved smooth muscle hyperplasia and thus inhibited AHR.[20] In the present study, we showed that endogenous and exogenous TRX1 was able to inhibit goblet cell hyperplasia in a murine asthma model induced by chronic exposure to OVA. In addition, rhTRX1 administration after the establishment of airway remodeling ameliorated goblet cell hyperplasia in this model. Our present and previous studies suggest that TRX1 has potential utility as a new therapy for patients with severe asthma resistant to treatment with corticosteroids.

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