

Original article

Thioredoxin reduces C-C chemokine-induced chemotaxis of human eosinophils

Background: Human thioredoxin (TRX) is one of redox-active proteins that regulate reactive oxidative metabolisms. In recent study, we found that serum levels of TRX were elevated in asthmatic patients with exacerbation; however, few details are known about the physiological role of TRX in allergic inflammation, involving eosinophil infiltration.

Objective: In the present study, we examined whether TRX modulated C-C chemokine-induced chemotaxis of human eosinophils.

Methods: Eosinophils were isolated from subjects with mild eosinophilia by modified CD16 negative selection. After incubation with or without recombinant TRX, chemotaxis of human eosinophils was measured using Boyden chamber.

Results: Preincubation with TRX suppressed eotaxin- and regulated on activation, normal T-cell expressed and secreted (RANTES)-induced chemotaxis of eosinophils. Although, TRX had no effect on the expression of C-C chemokine receptor 3, which is a receptor of eotaxin and RANTES, we demonstrated that the activation of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinases, which play an important role in eosinophil migration, was attenuated by the treatment with TRX.

Conclusion: Our results suggest that the elicited TRX is beneficial to reduce allergic inflammation through negative regulation of eosinophil functions and has potential in the treatment of allergic diseases, such as asthma.

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The pathogenesis of asthma is characterized by tissue inflammation, involving T lymphocytes and eosinophils (1) and chemotaxis of eosinophils is one of the most important events in the process of allergic inflammation. C-C chemokines such as eotaxin and regulated on activation, normal T-cell expressed and secreted (RANTES) are known as selective chemoattractants for eosinophils. The chemotactic response of eosinophils is mostly mediated by C-C chemokine receptor-3 (CCR3) (2), which is a receptor of eotaxin as well as RANTES and transduces signals eliciting Ca^{2+} influx, leading to chemotaxis through myosin light chain kinase activation by rearrangement of the actin cytoskeleton (3, 4).

Abbreviations: ADF, adult T-cell leukaemia-derived factor; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage; CCR, C-C chemokine receptor; ERK, extracellular signal-regulated kinase; FCS, foetal calf serum; HBSS, Hank's balanced salt solution; hrTRX, human recombinant thioredoxin; HTLV, human T-cell leukaemia virus; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; RANTES, regulated on activation, normal T-cell expressed and secreted; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; Th, T helper; TRX, thioredoxin.

Moreover, eotaxin activates extracellular signal-regulated kinase (ERK) 1/2 and p38 mitogen-activated protein (MAP) kinases in eosinophils and these kinases play an important role in eosinophil chemotaxis (5, 6).

Thioredoxin (TRX), which was originally identified as a hydrogen donor for ribonucleotide reductase in *Escherichia coli*, is a 12-kDa protein with a redox-active disulphide/dithiol (-Cys-Gly-Pro-Cys-) (7). Thioredoxin has a protective effect against oxidative stress by scavenging reactive oxygen species (ROS). Reactive oxygen species is also implicated in the pathogenesis of asthma, because it is produced from inflammation cells of asthma (8, 9). In particular, eosinophils migrate into the bronchial epithelium and release ROS as well as eosinophil granule proteins, which cause damage to the epithelium, leading to airway hyperresponsiveness (AHR) (10, 11). Moreover, our previous study reported that the levels of serum TRX elevates in asthma patients with attacks compared to those in remission (12).

A recent study reported that TRX suppresses AHR and allergic airway inflammation involving eosinophils and lymphocytes in a murine model of asthma (13). In addition, administration of TRX prevented the onset and

progression of acute respiratory distress syndrome (14), emphysema (15), cerebral ischaemia (16) and autoimmune myocarditis (17); thus, TRX is now recognized as a multifunctional protein. On the other hand, *in vitro*, some studies have reported that TRX, released from various cells in response to oxidative stress, has several cytokine and chemokine-like activities. For example, TRX activates monocytes and is a mitogenic cytokine for lymphocytes (18, 19). Moreover, TRX itself regulates the migration of neutrophils, monocytes and lymphocytes (20); however, very little is known about the functional role of TRX in the migration of human eosinophils.

In this study, to define the effect of TRX in regulating the C-C chemokine-stimulated migration of eosinophils, we examined whether TRX produces an effect on eotaxin and RANTES-induced migration of human purified eosinophils.

Methods

Eosinophil purification

Peripheral blood samples were obtained from subjects with mild eosinophilia. All subjects gave informed consent. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients as a modified method of Hansel et al. (21, 22). Furthermore, cells were purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) and then the cells (>99% purity) were suspended in Hank's balanced salt solution (HBSS; Life Technologies, Grand Island, NY, USA) with 1% foetal calf serum (FCS).

Chemotaxis assay

Chemotaxis of eosinophils was conducted in duplicate using 5- μ m polycarbonate, polyvinylpyrrolidone-free membranes in Boyden chambers. Human RANTES (10 nM; R&D Systems, Minneapolis, MN, USA) or eotaxin (10 nM; R&D Systems) was diluted in RPMI 1640 (Gibco, Grand Island, NY, USA) with 1% FCS and placed in the lower wells (100 μ l). Eosinophils were incubated with human recombinant thioredoxin (hrTRX), which was a gift from Dr Y. Nakamura (Kyoto University, Kyoto, Japan) (23) or medium for 1 h at 37°C and the cells were then washed twice. Aliquots (100 μ l) of the cell suspension at 2.0×10^5 cells/ml were placed in the upper chambers and the loaded chambers were incubated at 37°C in humidified air with 5% CO₂ for 1 h. The membrane was then removed, followed by fixation and staining for 3 min in May-Grünwald solution. Cells that migrated and adhered to the lower surface of the membrane were counted from 10 fields by light microscopy.

Analysis of CCR3 expression

To investigate CCR3 expression on human eosinophils, a FACScan flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) was used as previously described (24). Purified eosinophils (1×10^6 cells) were incubated with a fluorescein isothiocyanate-conjugate anti-human CCR3 monoclonal antibody (mouse IgG2, 5.0 μ g/ml; R&D Systems) or isotype-matched control

monoclonal antibody (5.0 μ g/ml; R&D Systems). The cells were washed and then the stained cells were analysed with a flow cytometer.

Measurement of intracellular calcium

For intracellular calcium measurement, purified eosinophils (10×10^6 cells in HBSS/HEPES without Ca²⁺) in suspension were loaded with 2 μ M fura 2-AM (Dojindo, Kumamoto, Japan) for 40 min at room temperature in the dark. After the eosinophils were washed in HBSS/HEPES with 4% FCS, the cells were resuspended at a concentration of 5×10^6 cells/ml in HBSS with Ca²⁺. Aliquots (50 μ l) of cells were dispensed into cuvettes and equilibrated with 1 mM calcium at room temperature for 10 min. Changes in fluorescence were measured using a Fluorescence Drug Screening System 2000 (Hamamatsu Photoics K.K., Hamamatsu, Japan) as previously described (25). Intracellular free calcium was calculated from fluorescence spectra (excitation wavelengths, 340 and 380 nm) in accordance with established methods (26). The eosinophils were incubated with 100 μ g/ml hrTRX or medium for 1 h at 37°C and then 10 nM eotaxin was added 90 s after commencing recording.

Western blot analysis

Purified eosinophils (1×10^6 cells) were treated with and without hrTRX (100 μ g/ml) for 1 h at 37°C followed by stimulation with and without eotaxin 10 nM for 2 min. The reaction was terminated by the addition of 15 volumes of ice-cold HBSS containing 1 mM Na₃VO₄. The cells were pelleted by centrifugation and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM Na₃VO₄, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1% Triton X-100, 10% glycerol and 1 μ g/ml aprotinin, leupeptin and pepstatin). After 20 min on ice, detergent-insoluble materials were removed by centrifugation at 4°C at 15 000 g. The supernatants were mixed with sodium dodecyl sulphate (SDS) sample buffer and boiled for 4 min. The SDS-polyacrylamide (10%) gels (Ready Gel J) were obtained from BIO-RAD (Tokyo, Japan). The electrophoresed gel was blotted onto Hybond ECL membranes (Amersham, Arlington Heights, IL, USA). Blots were incubated in blocking buffer containing 5% bovine serum albumin in TBS-T buffer (20 mM Tris-HCl, 137 mM NaCl and 0.05% Tween-20, pH 7.6) for 30 min followed by incubation in the primary 0.1 μ g/ml Ab (mouse mAb against phospho-p38 from Cell Signalling Technology; mouse mAb against phospho-ERK from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for the phosphorylation state of signalling proteins with gentle agitation overnight at 4°C. After washing three times in TBS-T buffer, blots were incubated for 30 min with a horseradish peroxidase (HRP)-conjugated secondary 0.04 μ g/ml Ab directed against the primary Ab. The blots were developed with the ECL substrate according to the manufacturer's instructions. Blots were subsequently reprobed with another Ab (rabbit polyclonal anti-p38 and anti-ERK2; Santa Cruz Biotechnology) for the nonphosphorylation state of signalling proteins after stripping in buffer of 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-ME, and 2% SDS at 56°C for 30 min. HRP-conjugated goat anti-mouse and anti-rabbit Abs were obtained from Santa Cruz Biotechnology.

Statistical analysis

Two groups of data were compared using Student's *t*-test. Other data were analysed by ANOVA using Dunnett's post-test. The *P*-value for significance was set at <0.05. All results are expressed as the mean $\pm$ SEM.

Results

TRX prevents RANTES and eotaxin-induced eosinophil chemotaxis

To determine the effects of TRX on eosinophil migration, purified human eosinophils were stimulated with hrTRX and then RANTES or eotaxin-induced chemotaxis was measured using Boyden chambers. Compared with vehicle, RANTES and eotaxin-induced eosinophil migration. On the other hand, preincubation of TRX with eosinophils significantly prevented C-C chemokine-induced migration (Fig. 1A, B).

TRX has no effect on CCR3 expression of eosinophil and eotaxin-induced calcium influx

A change in CCR3 expression is involved in the eosinophil chemotaxis; therefore, we examined whether TRX modulates the surface expression of CCR3 on eosinophils; however, flow cytometric study revealed that TRX had no effect on CCR3 surface expression (Fig. 2).

Calcium influx is also crucial for eosinophils to allow CCR3-mediated transmigration (4); therefore, we studied the effects of TRX in the calcium influx on eotaxin-induced eosinophil chemotaxis. No change in calcium mobilization was found when eosinophils were stimulated with vehicle instead of eotaxin (Fig. 3A). On the other hand, incubation with the medium of hrTRX resulted in a marked increase in eotaxin-induced calcium mobilization (Fig. 3B). Preincubation with hrTRX for 1 h did not suppress the increase of calcium mobilization (Fig. 3C).

TRX inhibits the MAPK signalling pathway in eotaxin-induced eosinophil chemotaxis

The MAPK signalling pathway was studied to explore inhibition mechanisms further by TRX in agent-induced migration. To investigate MAPK pathway activity,

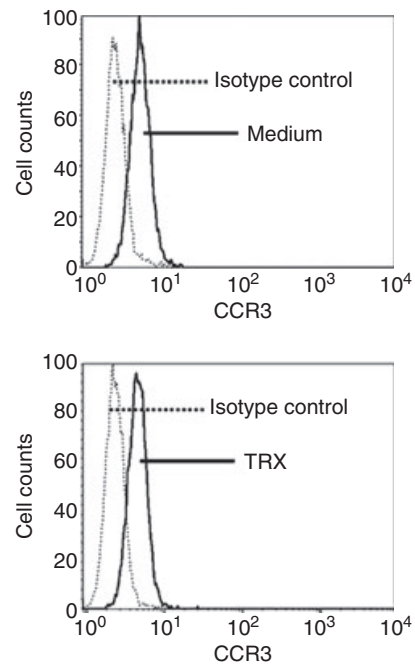


Figure 2. Examination of the effect of TRX on CCR3 surface expression in eosinophils. Purified eosinophils were incubated with 100 µg/ml hrTRX or medium alone for 1 h. After incubation, eosinophils were treated with monoclonal antibody directed against CCR3. Representative histograms are shown. Cells stained with isotype-matched antibody were used for negative control staining. No significant difference in the surface expression of CCR3 was observed between eosinophils stimulated with hrTRX and medium ($67.94 \pm 0.08\%$ vs $68.06 \pm 1.77\%$ CCR3-positive cells). The results are representative of experiments with similar results using eosinophils from three different donors.

eosinophils were stimulated with eotaxin followed by electrophoresis and Western blotting with anti-p-ERK or anti-p-p38 Ab. Treatment with 100 µg/ml TRX partially

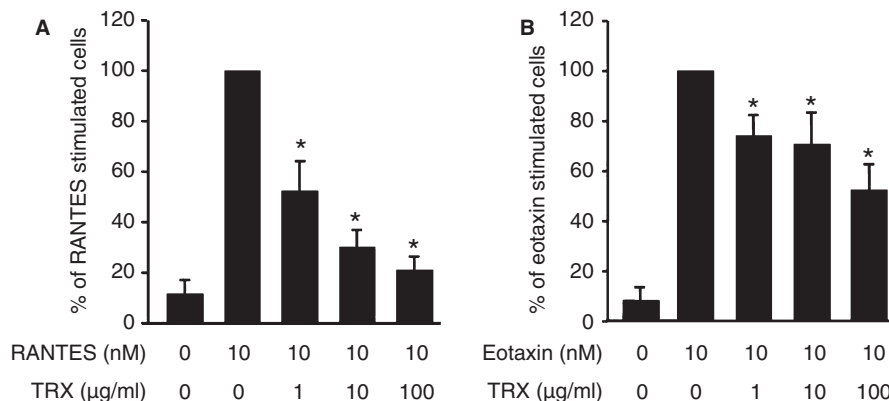


Figure 1. Effect of TRX on eosinophil chemotaxis stimulated with 10 nM RANTES (A) or 10 nM eotaxin (B). Purified eosinophils preincubated with increasing concentrations of hrTRX for 1 h. Migration assays were performed using Boyden chambers. Chemotactic response to each agent was considered as 100%. Data are expressed as the mean of eight experiments $\pm$ SEM. * $P < 0.05$ vs control (medium; agent alone).

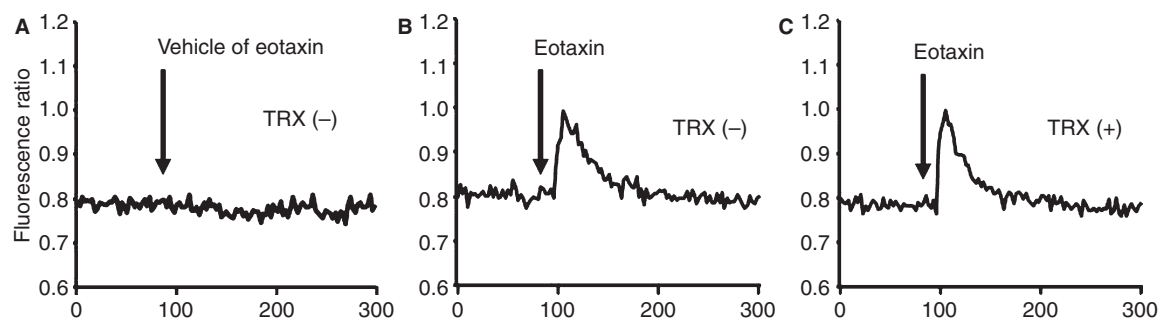


Figure 3. Effect of TRX on eotaxin-induced intracellular calcium flux. Purified eosinophils loaded with 2 μ M of fura-2 were pre-equilibrated with Ca^{2+} . Eosinophil preparations were then stimulated with medium or 100 μ g/ml hrTRX. After 1 h of incubation, eotaxin (10 nM) or vehicle was added (solid arrows). Changes in intracellular free-calcium levels were detected as an increase in fluorescence intensity of calcium-sensitive dye fura-2. (A) Negative control, (B) eosinophils treated with medium, (C) eosinophils treated with hrTRX. The results are representative of experiments with similar results using eosinophils from three different donors.

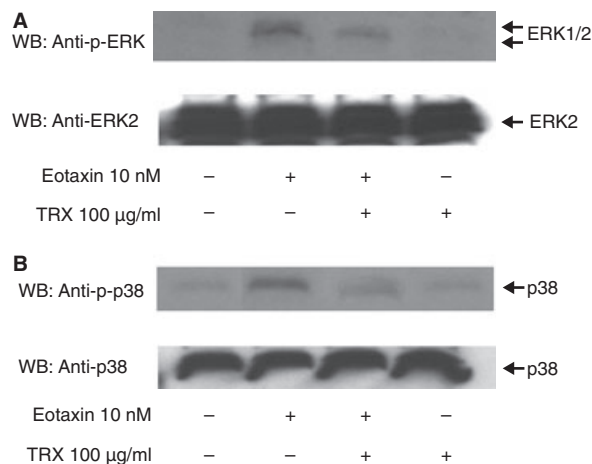


Figure 4. Examination of the effect of the intracellular signalling pathway on the inhibitory effect of TRX. Purified eosinophils were incubated with or without 100 μ g/ml TRX for 1 h. After incubation, eosinophils were stimulated with eotaxin (10 nM) for 2 min. Phosphorylated ERK1/2 (p-ERK) and total ERK2 (ERK2), and phosphorylated p38 MAPK (p-p38) and total p38 MAPK (p38) were determined by Western blot analysis. HrTRX partially abrogated the eotaxin-induced phosphorylation of ERK (A) and p38 (B). Experiments were repeated at least three times with a different cell preparation.

inhibited eotaxin-induced phosphorylation of ERK1/2 as well as p38 (Fig. 4A, B). The membranes were reprobed with anti-ERK2 or anti-p38 Ab incubated with the same amounts of proteins loaded on the gels.

Discussion

In the pathogenesis of allergic inflammation, including asthma, the migration of eosinophils to inflammatory sites is an important step before eosinophils release toxic granule proteins and ROS into tissues. In the present study, we investigated the functional role of TRX in

human eosinophil chemotaxis. Our present data demonstrated that TRX attenuated eosinophil migration towards both eotaxin and RANTES. To our knowledge, this is the first report showing TRX ability in C-C chemokine-induced function of eosinophils.

Previously, we reported that levels of serum TRX elevate in asthma patients with attacks compared to those in remission (12), suggesting that TRX may be beneficial to reduce oxidative stress and allergic inflammation. Indeed, Ichiki et al. (13) found that TRX administration suppressed AHR and eosinophil accumulation in bronchoalveolar lavage fluid (BALF) of asthmatic model mice and increased interleukin (IL)-1 β , T helper 1 (Th1) cytokine, as well as IL-18, which induce IL-12-driven Th1 development (27). Th2 cytokines (e.g. IL-4 and IL-5) regulate eosinophilic airway inflammation and AHR (28) and are prevented by Th1 cytokines. Thus, they state that TRX may suppress eosinophil accumulation in the airway and AHR through the inhibition of Th2 cytokine production by TRX-induced Th1 cytokines (13). On the other hand, in this study, TRX attenuated the C-C chemokine-induced chemotaxis of human eosinophils in the absence of Th1 or Th2 cytokines (Fig. 1). Human TRX produced by human T-cell leukaemia virus-1 (HTLV-1)-transformed T-cells was cloned as adult T-cell leukaemia-derived factor (ADF). We also found that, although the level of RANTES in BALF of HTLV-1-infected subjects increased compared to healthy subjects, few eosinophils were detected in BALF of the patients. This may also suggest that TRX/ADF released from HTLV-1-infected T-cells inhibited RANTES-induced migration of eosinophils (data not shown, manuscript in preparation, Junichi Chihara). These data may indicate that TRX negatively regulates allergic airway inflammation by eosinophils through not only the suppression of Th2 cytokines but also direct involvement in eosinophil chemotaxis.

Regarding the chemotaxis of eosinophils, a previous study reported that TRX itself induced the migration of eosinophils from patients with hypereosinophilia (29).

In our study, preincubation with TRX significantly attenuated eotaxin and RANTES-induced chemotaxis. Consistent with our results, in monocyte migration, Pagliei et al. (30) have reported that TRX specifically cross-desensitizes monocytes to MCP-1. Moreover, in neutrophil migration, Nakamura et al. (23) found that TRX suppresses lipopolysaccharide (LPS)-induced neutrophil chemotaxis, whereas TRX is itself chemotactic for neutrophils (20). These results are not contradictory, because some studies have reported that preincubation with chemokines inhibits chemotaxis (31, 32); therefore, TRX may also have the ability to migrate leucocytes and to suppress other chemokine-induced migration of leucocytes. Thioredoxin did not influence cell viability in the concentration range used in this study (data not shown). Thus, further studies are necessary to clarify the physiological capacity of TRX.

C-C chemokine receptor 3 is a receptor of eotaxin and RANTES and upregulation of CCR3 surface expression on eosinophils augments chemokine-induced migration (33). In this study, TRX had no effect on the expression of the receptor (Fig. 2) and it may be assumed that TRX regulates the downstream signalling pathway of eotaxin/CCR3. Furthermore, although the calcium mobilization

induced by chemokines is a regulator of eosinophil migration (3, 4), TRX failed to suppress the eotaxin-induced calcium response (Fig. 3). On the other hand, TRX attenuated eotaxin-stimulated activation of ERK1/2 and p38 MAPK pathways (Fig. 4), which play an important role in cell chemotaxis (5, 6). This result may suggest that TRX modulates the downstream signalling of CCR3 to attenuate factor-induced migration of human eosinophils. Consistent with our finding, Ichiki et al. (13) have found that preincubation with TRX prevents LPS-induced chemotaxis of neutrophils by the suppression of p38 MAPK phosphorylation.

In conclusion, we have found that the level of serum TRX in asthmatic patients elevates during exacerbation. Moreover, this present study demonstrated that TRX attenuates C-C chemokine-induced chemotaxis of human eosinophils and furthermore, the effect of TRX may be caused by inhibition of the MAPK signalling pathway, which is important in eosinophil chemotaxis. These findings may indicate that, in asthma, TRX elicited *in vivo* is beneficial to reduce allergic inflammation, therefore providing further insights into the functional role of TRX in allergic reaction and suggesting that TRX has potential in the treatment of allergic diseases, such as asthma.

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