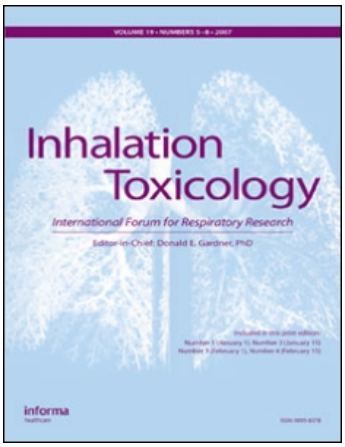

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Bronchial epithelial cells exposed to allergens typically secrete chemokines to recruit eosinophils. Persistent inflammation and repair responses result in airway remodeling and irreversible airflow limitation. House dust mite (HDM) is a common allergen causing allergic disorders. Thioredoxin (TRX) is a redox protein that scavenges reactive oxygen species (ROS). This study was to elucidate how TRX mediates gene expression of remodeling factors of human bronchial epithelial cells in response to HDM stimuli interacting with eosinophils. This study cultured normal human bronchial epithelial (BEAS-2B) cells with eosinophils exposed to 0.5 μg/ml recombinant *Dermatophagoides pteronyssinus* 1 (rDer p1) protease to mimic the allergen-immune reaction. Eosinophils were induced by rDer p1 protease to secrete tumor necrosis factor (TNF)-α and generate ROS. When cultured with rDer p1-stimulated eosinophils, BEAS-2B cells released interleukin-6 and underwent apoptosis. The HDM-stimulated eosinophils applied oxidative stress and apoptosis to BEAS-2B cells through the release of mediators. Damaged BEAS-2B cells interfered with gene expression of remodeling factors, such as transforming growth factor (TGF)-β1, epidermal growth factor receptor (EGFR), cyclin dependent kinase inhibitor ($p21^{waf}$) and matrix metalloproteinase (MMP) 9, relevant to inflammatory response and epithelial repair in airway remodeling. Notably, BEAS-2B cells over-expressing TRX reduced eosinophil-derived apoptosis and suppressed underlying airway remodeling via attenuation of TGF-β1, EGFR and $p21^{waf}$ and up-regulation of MMP9 expression. Results of this study indicated TRX-over-expressing bronchial epithelial cells attenuated TGF-β1 and activated MMP9 expression to prevent airway remodeling from HDM-induced inflammation. The finding can be as a reference for further therapeutic studies of TRX.

INTRODUCTION

Allergic disorders, such as asthma, allergic rhinitis and eczema, are the most common chronic diseases among children, affecting 150 million individuals worldwide (Beasley, 2002). The prevalence of asthma in children has increased dramatically in recent decades. Sloughed epithelial cells are typically identified in the sputum of asthmatic patients as well as patches of denuded epithelium in airway biopsies (Cohn et al., 2004). Epithelial damage and activation may in part be the result of chronic inflammatory stimuli that prolong the period of epithelial repair. Persistent allergen-induced inflammation is accompanied by structural changes in bronchial epithelium (i.e., airway remodeling) in asthmatic patients. These structural changes in the epithelium are suggested to be responsible for airway wall thickening, airway flow limitation, and airway hyperresponsiveness (Pascual and Peters, 2005). Furthermore, the respiratory epithelium is believed to orchestrate airway remodeling through aberrant production of growth factors and matrix metalloproteinases (MMPs) (Hamilton et al., 2003). Various stimuli in the airway, including allergens (i.e., house dust mite), inflammatory cells (such as eosinophils, activated T cells, mast cells and macrophages), and structural tissue cells (i.e. airway epithelium, endothelial cells and fibroblasts) may participate in regulating this response (Rennard, 1996). Eosinophils dysregulate extracellular matrix homeostasis caused by fibroblast interleukin (IL)-6 secretion implicating in fibrogenesis for consequent tissue remodeling (Gomes et al., 2005).

The bronchial epithelium provides a protective barrier against external environments. Activated airway epithelial cells are a source of hematopoietic cytokines, pro-inflammatory cytokines, and chemokines (Cohn et al., 2004). Chemokines release may

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recruit granulocytes, such as eosinophils and neutrophils, to the respiratory tract. Eosinophils are inflammatory effector cells that accumulate at the site of allergic inflammation. Catalysis reactions in eosinophils generate reactive oxygen species (ROS) in the lung; these are used primarily for host defense against parasites and pathogens, and even contribute to airway inflammation and damage in bronchial tissues (Andreadis et al., 2003). Since eosinophils express protease-activated receptor (PAR) 2, neutrophil-derived serine proteases activate eosinophils to produce superoxide, pro-inflammatory cytokines and neutrophilotactic chemokines and may further aggravate airway inflammation (Hiraguchi et al., 2008).

House dust mite (HDM) is an allergen that increases the incidence of allergic diseases (Busse and Rosenwasser, 2003). *Dermatophagoides pteronyssinus* 1 (Der p1) is the major mite allergen; its cysteine protease activity cleaves tight junction adhesion proteins (such as zona occuldens protein 1), increases the permeability of allergens in the respiratory tract, and disrupts regulation of IgE synthesis (Kalsheker et al., 1996; Wan et al., 2001). Notably, Der p1 also induces the release of pro-inflammatory mediators, e.g., IL-6, IL-8, granulocyte monocyte colony stimulating factor (GM-CSF) and RANTES (regulated upon activation, normal T-cell expressed and secreted) protein from bronchial epithelial cells due to its proteolytic activity and activation of nuclear factor (NF)-κB and activator protein (AP)-1 (King et al., 1998; Stacey et al., 1997; Adam et al., 2006). These mediators trigger the accumulation of inflammatory cells and thus persist chronic allergic inflammation in the airway; they also activate NF-κB in human eosinophils, leading to increased secretion of GM-CSF, tumor necrosis factor (TNF)-α and IL-8 (Coward et al., 2004). Additionally, Der p1 induces the release of inflammatory cytokines and expression of adhesion molecules in a co-culture system of human eosinophils and bronchial epithelial cells (Wong et al., 2006). Der p1 stimulates the cytokine expression, superoxide production and degranulation in airway epithelial cells via a PAR2-independent mechanism, and directly activate eosinophils (Adam et al., 2006).

Thioredoxin (TRX) is a 12-kDa redox (reduction/oxidation)-active protein with a highly conserved disulfide site (-Cys-Gly-Pro-Cys-) used for scavenging reactive oxygen (Masutani et al., 2005). TRX regulates the cellular redox balance, promotion of cell growth, inhibition of apoptosis, and modulation of inflammation (Burke-Gaffney et al., 2005; Ichiki et al., 2005; Kaimul Ahsan et al., 2005). Airway remodeling and eosinophil infiltration is prevented in Balb/c:human TRX transgenic mice chronically exposed to ovalbumin (OVA) (Imaoka et al., 2007). Administration of TRX improves airway pathology during airway remodeling.

Airway remodeling is induced by cytokines and mediators for tissue repair resulting from chronic and/or short-term exposure to inflammatory stimuli. This remodeling causes irreversible airflow limitation and increases airway hyperresponsiveness (James et al., 1989). Once transformed, the remodeled lung resists asthma therapy (Yamauchi and Inoue, 2007); thus, early intervention must be considered to retard any onset and/or progression of airway remodeling. The HDM is a common allergen associated with allergic disorders due to T helper (Th) 2-meidated chronic inflammation, and the major effector Th2 cells are eosinophils rather than Th1 neutrophil cells (Wardlaw et al., 2002). However, it remains unknown whether Der p1-activated eosinophils induce expression of remodeling factors in damaged bronchial epithelial cells. This study sought to examine the gene expression of remodeling factors in human bronchial epithelial cells exposed to HDM upon eosinophils interaction, and to investigate how TRX might mediate this phenomenon.

MATERIALS AND METHODS

House Dust Mite rDer p1

A recombinant mite protease of *Dermatophagoides pteronyssinus*, rDer p1, constructed from *Pichia pastoris* eukaryotic yeast was used in this study (Indoor Biotechnologies Ltd, Manchester, UK). The stock solution of 1.8 mg rDer p1/ml was prepared (carrier free) in phosphate-buffered saline (PBS; pH 7.2) and filtered through a 0.22 mm filter. The stock solution of rDer p1 was then serially diluted with culture medium for use in the various experiments.

TRX Plasmid DNA Construction and Cloning

Human TRX cDNA was prepared by PCR based on the sequence derived from the NCBI (NM_003329). The TRX was flag-tagged in a p3XFlag-CMV-14 vector (between the restriction sites BamHI and SalI) according to the manufacturer's recommended protocol (Sigma, St. Louis, MO). After sequence verification, the C-terminal of the TRX gene attached 3flag was inserted into a tetracycline-controllable pTRE$_2$hyg vector (BD Biosciences, San Jose, CA) through restriction enzymes catalysis of ClaI and SalI (for identification of exogenous TRX expression). The plasmid pTRE$_2$hyg-TRX-3flag was then transformed into DH5α competent cells (Invitrogen, Carlsbad, CA). The presence of the plasmid DNA was verified by restriction endonuclease digestion and agarose gel electrophoresis.

Development of Tet-on Human TRX-Inducible BEAS-2B Cells

The exogenous pTRE$_2$hyg-TRX-3flag plasmid DNA was transfected into an established Tet-on regulator expression BEAS-2B cells (normal human bronchial epithelial cells; ATCC CRL-9609) by lipofectamine (Invitrogen) and OPTI-MEM I medium (Invitrogen) following the manufacturer's protocol. After G418 selection, the BEAS-2B Tet-on pTRE$_2$hyg-TRX-3flag cells were developed, and over-expressed TRX (via Tet-on system) switched on by 2 μg/ml doxycycline (Sigma) for 24 h. The TRX-over-expressed cells (TRX-TD; BEAS-2B cells over-expressing pTRE$_2$hyg-TRX-3flag via Tet-on system induced by doxycycline) were examined for the expression of human flag mRNA and protein, respectively, by quantitative real time PCR and flow cytometric analysis.

Intracelluar TRX-Flag Immunostaining

The expression of exogenous transfected TRX was monitored by intracellular flag immunostaining. BEAS-2B and TRX-TD cells were respectively fixed by commercial fixation solution (eBioscience, San Diego, CA) while vortexing. The cells were then incubated in the dark at room temperature for 20 min and permeabilized for staining using permeabilization buffer (eBioscience). The cells then received an optimal concentration of monoclonal M2 anti-flag-FITC antibody (Ab) (Sigma) and analyzed by flow cytometry (CyFlow, Partec, Münster, Germany) using WinMDI 2.8 software.

Isolation of Human Primary Eosinophils and Matured HL-60/clone 15 Eosinophils

Venous blood of HDM-atopic volunteers (eight males, 18-30 years old) with their informed consent was anti-coagulated with EDTA, and eosinophils were isolated from their peripheral mononuclear leukocytes by a magnetic-activated cell sorting (MACS) kit (Miltenyi Biotec, Bergisch Gladbach, Germany). After 45 min sedimentation with 6% dextran (Sigma), leukocyte-rich plasma (LRP) was recovered and diluted with calcium- and magnesium-free PBS (Gibco Laboratories, Grand Island, NY). The LRP suspension was then overlaid onto 5 ml Ficoll-Hypaque (density = 1.077 g/ml, Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged at 2000 rpm (20 min, 25°C). The granulocyte/red blood cell (RBC)-bearing pellet was recovered, and erythrocytes were removed using cold RBC-lysis buffer. The remaining granulocytes were washed twice with cold autoMACS^{TM} running buffer (Miltenyi Biotec), and the total cell number was then determined. The isolated granulocytes were resuspended, and the non-eosinophils were labeled with 20 μl biotin-Ab cocktail and 40 μl anti-biotin microbeads (per 10^7 total cells). After incubation overnight at 4°C, the granulocytes were loaded onto a separation column positioned in the strong magnetic field of a MACS separator, and untouched eosinophils were depleted from magnetized microbeads and then eluted with running buffer.

Human undifferentiated eosinophil HL-60/clone 15 cells (ATCC CRL-1964) were grown in culture medium containing RPMI (Invitrogen), 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (Sigma). Cells were induced to differentiate to eosinophil-like cells by treatment with medium containing 0.5 μM butyric acid (Sigma) for 7 days.

Isolated human primary eosinophils (hEOS) and differentiated HL-60/clone 15 eosinophils (EOS/HL-60) were confirmed for purity by analysis of cytospin smears treated with Liu's stain. The purity of the eosinophils was > 80%; viability of the isolated eosinophils was assessed by trypan blue staining. Purified hEOS and EOS/HL-60 cells were then washed twice with PBS and suspended in culture medium.

Cell Culture of Human Bronchial Epithelial Cells and Eosinophils

The co-culture of human airway cells and eosinophils was performed to mimic the *in vivo* immune response to mite exposure. In the co-culture, primary eosinophils or differentiated HL-60/clone 15 eosinophils were stimulated with 0.5 μg/ml rDer p1 for prior 24 h. The medium containing stimulated eosinophils and rDer p1was then collected and the human bronchial epithelial cells cultured in this medium. In the treatment of airway cells with conditioned medium (i.e., non-co-culture scenario), eosinophils were stimulated by rDer p1 for 24 h and the medium recovered for use in treating human bronchial epithelial cells for 24 h. Both attached and detached airway cells were collected by centrifugation for later analyses.

Both human bronchial epithelial BEAS-2B and TRX-TD cells were grown on coated tissue culture plates (a mixture of 0.01 mg/ml fibronetin, 0.03 mg/ml vitrogen 100, and 0.01 mg/ml bovine serum albumin in 10 ml LHC-9 medium) in LHC-9 medium (Invitrogen) at 37°C with 5% CO_2. Alternatively, the airway cells were cultured in eosinophil RPMI culture medium generated using hEOS or EOS/HL-60 cells.

Determination of Supernatant IL-6 and TNFα Protein

The medium from the cultured cells was collected and centrifuged at 1,500 rpm (10 min, 4°C). The concentration of IL-6 and TNFα protein secreted by the bronchial epithelial cells was determined using corresponding ELISA Ready-SET-Go kits (eBioscience). The optical density in each kit well was detected using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA) and levels of each cytokine deduced from the absorbance value by extrapolation from a standard curve generated in parallel.

ROS Determination

Cells were rinsed twice with PBS and incubated with 10 μM CM-H_2DCFDA (cloromethyl dihydrodichlorofluorescein diacetate; Molecular Probes, Invitrogen) for 30 min. CM-H_2DCFDA is a non-fluorescent compound when reduced, and emits fluorescence when oxidized by ROS. The adherent cells were trypsinized, washed with PBS three times, and then resuspended in 1 ml PBS. The fluorescent signal (reflecting ROS) was then determined by flow cytometry (CyFlow, Partec). Data analysis was performed using WinMDI 2.8 software.

Apoptosis Determination

The level of apoptosis was determined using an Annexin V-FITC apoptosis detection kit (R & D Systems Inc., Minneapolis, MN). This analysis was performed based on a previous procedure (Berkova et al., 2006) with minor modification. The trypsinized cells were washed twice with cold PBS and resuspended in 1X binding buffer. Cell suspension (100 μl) was treated with 5 μl of Annexin V and 10 μl of PI (propidium iodide) solutions and the cells then incubated at 4°C in the dark

for 15 min before adding 400 μl of binding buffer. The cells then underwent flow cytometric analysis for the presence of the fluorescent signal.

Quantitative Real-Time PCR

Total RNA was extracted with Trizol reagent (Invitrogen), and 3 μg RNA was reverse transcribed with RT random primers and M-MLV reverse transcriptase. Subsequently, a fragment of 100 ng cDNA was amplified by PCR with 40 cycles of denaturing (95°C, 15 sec), annealing (55°C, 30 sec) and extension (72°C, 45 sec) using 2X power SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA). PCR primers: TRX sense 5′-GGA CGC TGC GGG TGA TA-3′ and anti-sense 5′-GAG AGG GAA TGA AAG AAA GGC TT-3′; TGF-β1 sense 5′-ACA ATT CCT GGC GAT ACC TCA-3′ and anti-sense 5′-GGC GAA AGC CCT CAA TTT C-3′; EGFR sense 5′-GGA GAG GAG AAC TGC CAG AAA CT-3′ and anti-sense 5′-GCA GCC TGC AGC ACA CTG-3′; $p21^{waf}$ sense 5′-GGG ACA GCA GAG GAA GAC CAT-3′ and anti-sense 5′-GGA GTG GTA GAA ATC TGT CAT GCT-3′; MMP9 sense 5′-ACC ACC TCG AAC TTT GAC AGC-3′ and anti-sense 5′-TCT AAG CCC AGC GCG TG-3′; β-actin sense 5′-TTG TTA CAG GAA GTC CCT TGC C-3′ and anti-sense 5′-ATG CTA TCA CCT CCC CTG TGT G-3′. Monitoring and quantitative analysis of PCR products were carried out by a sequence detector (Model 7300, Applied Biosystems) according to the manufacturer's instructions. The signal of SYBR green was measured at 530 nm during extension phase, and collected and analyzed with SDS 1.0 software. The threshold cycle (Ct) value denotes the cycle number at which the fluorescence generated within a reaction across the threshold, thus the Ct value is at the point accumulated a sufficient number of amplicons during the reaction. The relative level of mRNA expression is a ratio of optical density of the experimental groups to that of β-actin (internal control, an endogenous house-keeping gene). The relative Ct value of different condition was compared to that of control cells as reference to estimate the fold change of mRNA expression among the samples. Replicates were performed for each primer pair.

Western Blot Determination of MMP9 Protein

BEAS-2B and TRX-TD cells were individually cultured with rDer p1-stimulated eosinophils neutralized with/without anti-TGF-β1 Ab (1 μg/ml; R & D Systems). Cell lysate was then prepared using protein extract buffer containing 0.6 M KCl, 1% Triton X-100, 0.02 M Tris-HCl (pH 7.0), 1.0 mM phenylmethylsulfonyl fluoride, and 50 μg/ml aprotinin (all from Sigma), and centrifuged at 12,000 rpm (3 min, 4°C). Protein samples in the supernatant were immediately transferred to a clean tube, and the concentration assessed using a DC protein assay kit (Bio-Rad, Hercules, CA). Proteins in the samples were then electrophoresed over a 12.5% sodium dodecyl sulfate-polyacrylamide gel, and subsequently transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membrane-bound proteins were then immunostained with anti-MMP9 Ab (R & D Systems) and anti-β-actin mAb (Cell Signaling, Danvers, MA), followed by treatment with secondary anti-IgG HRP Ab (Chemicon, Billerica, MA). Any tagged proteins were then detected using a chemiluminescence reagent (Perkin Elmer, Boston, MA) and photographed in a G:Box ChemiXT 16 system (Syngene, Frederick, MD).

Statistical Analysis

Results were described as mean ± standard deviation. All statistical analysis was conducted by the statistical package SPSS13.0. The differences were investigated using Student's t-test and one-way analysis of variance (ANOVA). A two-tailed p-value < 0.05 was considered significant.

RESULTS

Dose- and Time-Dependent IL-6 Secretion of Human Bronchial Epithelial Cells with rDer p1 Stimulation

Preliminary experiments were performed to determine whether the recombinant mite protease rDer p1 could cause BEAS-2B cells to secrete IL-6. The results showed that BEAS-2B cells were significantly induced to secrete IL-6 in dose-dependent manner (Figure 1A); secretion was significant using rDer p1 levels above 0.5 μg/ml for the 24 h period.

Time-course experiments were performed in BEAS-2B cells treated with 0.5 μg/ml rDer p1 for periods from 0 to 48 h (Figure 1B). The results showed that IL-6 release in rDer p1-stimulated BEAS-2B cells was time-dependent. After 8 h, levels of release by the stimulated cells were significant. There was no observable cell death caused by the mite rDer p1 protease in this study.

IL-6 Secretion by Human Bronchial Epithelial Cells Cultured with Eosinophils Exposed to rDer p1

BEAS-2B generally secreted IL-6 at a basal level of 144.4 ± 3.8 pg/10^6 cells (Figure 2). Mite rDer p1 protease at 0.5 μg/ml significantly activated confluent BEAS-2B to release IL-6 (350.4 ± 25.2 pg/10^6 cells). BEAS-2B cells cultured with hEOS or differentiated EOS/HL-60 cells increased their release of IL-6 (541.7 ± 13.8 and 456.5 ± 30.0 pg/10^6 cells). With co-culture of rDer p1-stimulated hEOS or differentiated EOS/HL-60 cells, BEAS-2B cells displayed even further increases in IL-6 secretion (810.4 ± 9.5 and 687.0 ± 41.1 pg/10^6 cells).

TNFα Level of Human Bronchial Epithelial Cells and Eosinophils

TNFα production by BEAS-2B cells with and without 24-h rDer p1 stimulation was 4.92 ± 0.17/10^6 cells and 6.16 ± 0.63 pg/10^6 cells, respectively (Figure 3). Higher TNFα levels were found in the supernatant of rDer p1-stimualted EOS/HL-60 cells and BEAS-2B cells co-cultured with rDer p1-stimulated EOS/HL-60 cells (16.04 ± 0.75/10^6 cells and 14.86 ± 1.25 pg/10^6 cells, respectively).

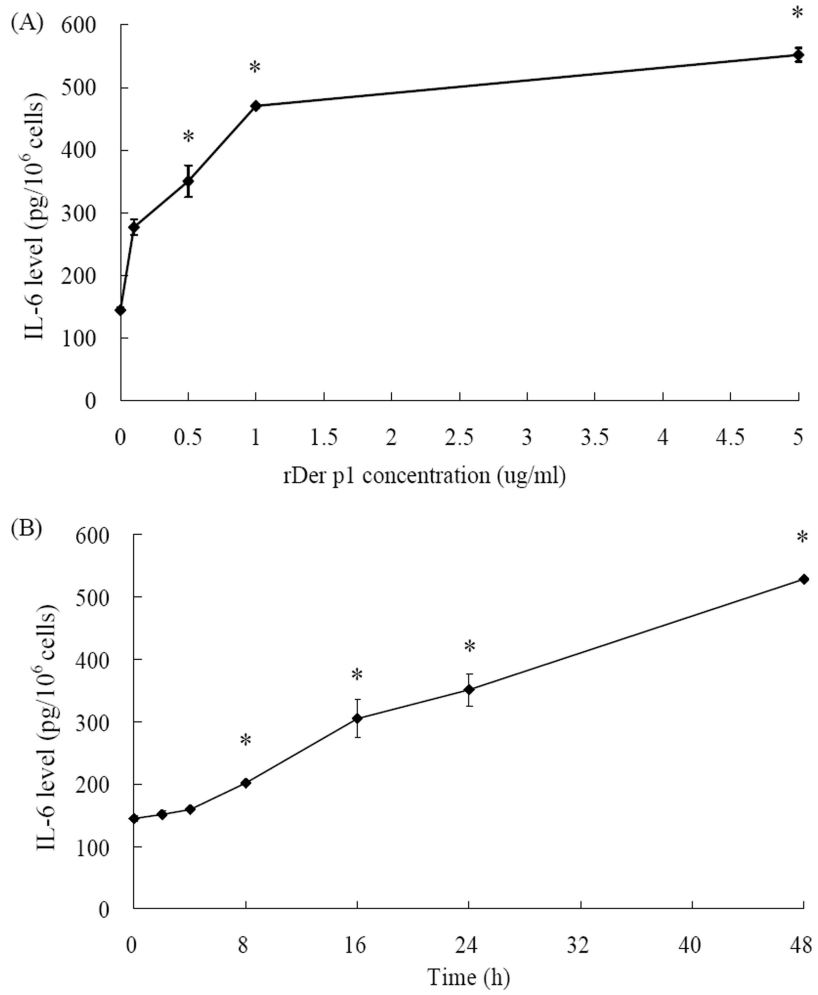

FIG. 1. IL-6 secretion of human bronchial epithelial BEAS-2B cells with rDer p1 stimulation in dose- and time-dependent manner. (A) BEAS-2B cells had significantly increased IL-6 secretion with higher than 0.5 μg/ml rDer p1 stimulation for 24 h. (B) Confluent BEAS-2B cells treated 0.5 μg/ml rDer p1 were with significant increases after 8 h. Differences were statistically analyzed by Student's t-test. Asterisk indicates significant at $p < 0.05$ compared to the sham experiments that BEAS-2B cells cultured in LHC-9 medium.

Thioredoxin Expression in Human Bronchial Epithelial Cells

TRX-TD cells, human TRX-inducible Tet-on BEAS-2B cells over-expressing TRX, were developed to examine whether the anti-oxidation role of TRX mediated the oxidative damage induced by the HDM-stimulated eosinophils in human bronchial epithelial cells. Using real-time PCR analysis, it was seen that the expression of total TRX mRNA in TRX-TD cells was significantly (4.3-fold) higher than in the BEAS-2B cells (Figure 4A). Via analysis of intracellular anti-human flag FACS, it was noted that TRX-TD cells had a higher amount of flag-tagged TRX protein than the BEAS-2B cells (intensity: 6.0 ± 0.6 *vs.* 15.4 ± 1.9) (Figure 4B).

ROS Generation by Eosinophils and Human Bronchial Epithelial Cells

Differentiated EOS/HL-60 cells were significantly induced to generate ROS after 2-h rDer p1 exposure (26.2 ± 5.0 %); however, these stimulated levels declined somewhat after 24 h of exposure (9.2 ± 5.0%) (Figure 5A). Differentiated EOS/HL-60 cells were not induced to generate ROS when cultured with confluent BEAS-2B cells (7.0 ± 1.2%). In contrast, after 24-h rDer p1 stimulation, differentiated EOS/HL-60 cells co-cultured with BEAS-2B cells displayed sustained ROS generation over a further 24 h (47.8 ± 3.5%). Regarding the bronchial epithelial cells themselves, ROS generation was induced in BEAS-2B cells cultured with rDer p1-stimulated EOS/HL-60

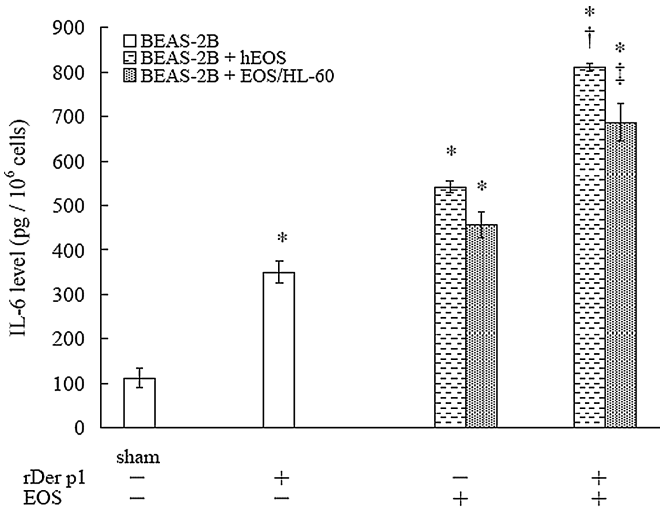

FIG. 2. IL-6 secretion of BEAS-2B cells cultured with human eosinophils in exposure to rDer p1 protease. Confluent BEAS-2B cells (10^6 cells) were individually cultured with human primary eosinophils (hEOS; 10^5 cells) or differentiated HL-60/clone 15 eosinophils (EOS/HL-60; 10^6 cells) with/without the prior 24-h 0.5 μg/ml rDer p1 stimulation. BEAS-2B cells significantly had increased IL-6 released in the treatment of rDer p1 and eosinophils co-culture. * indicates significant at $p < 0.05$ compared with sham BEAS-2B cells in RPMI medium, † and ‡ at $p < 0.05$ compared with BEAS-2B cells co-cultured respectively with hEOS or EOS/HL-60 cells in RPMI medium.

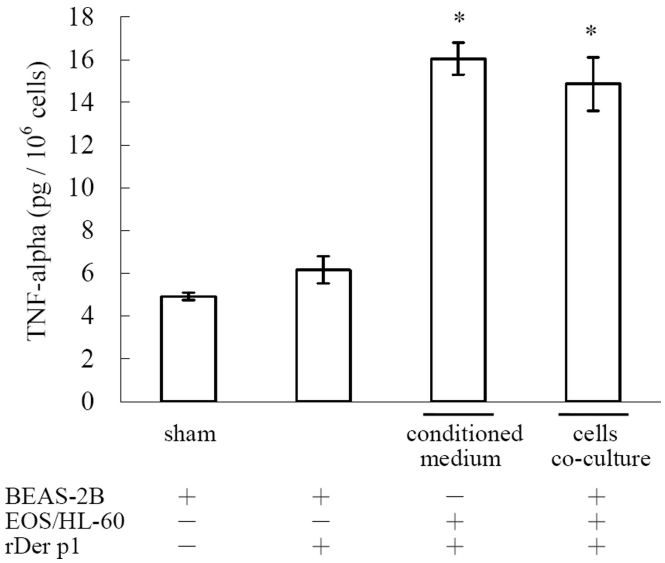

FIG. 3. Supernatant TNFα level of BEAS-2B cells and eosinophils in exposure to rDer p1. The conditioned medium was the supernatant of differentiated EOS/HL-60 cells treated 0.5 μg/ml rDer p1 for 24 h. In cells co-culture, confluent BEAS-2B cells cultured with EOS/HL-60 cells and rDer p1 for 24 h. The supernatant of rDer p1-stimualted EOS/HL-60 cells contained the higher TNFα level. It suggested EOS/HL-60 cells secreted the mediator protein TNFα after rDer p1 exposure. * indicates significant at $p < 0.05$ compared with sham BEAS-2B cells in RPMI medium.

cells (15.8 ± 0.1 %) but not in TRX-TD cells (7.1 ± 3.2%) (Figure 5B).

Suppression of Apoptosis in Human TRX-Over-Expressing Bronchial Epithelial Cells upon Interaction with rDer p1 and Eosinophils

Human BEAS-2B cells did not undergo apparent apoptosis after rDer p1 stimulation or co-culture with eosinophils (5.3 ± 0.1% and 4.8 ± 1.1%) (Figure 6). Even a higher dose of rDer p1 (5 μg/ml) had no effect of apoptosis in BEAS-2B cells (5.4 ± 1.1 %) (data not shown). To further examine whether eosinophil-derived mediators affected the bronchial epithelial cells, BEAS-2B and TRX-TD cells were individually cultured with the cell-free conditioned medium from the stimulated EOS/HL-60 cells previously activated by 0.5 μg/ml rDer p1 for 24 h, or co-cultured directly with the eosinophils. Apoptosis occurred in BEAS-2B cells cultured with the rDer p1-stimulated EOS/HL-60 cells (9.0 ± 3.1%) or its conditioned medium (12.1 ± 2.2%). Furthermore, over-expression of human TRX in the TRX-TD cells suppressed these pro-apoptotic outcomes (i.e. only a level of 5.4 ± 0.8% in cells that received conditioned medium).

Remodeling Factor Gene Expression in Human Bronchial Epithelial Cells upon Interaction with rDer p1 and Eosinophils

The influence of rDer p1 and eosinophils on gene expression of remodeling factors, TGF-β1, EGFR, $p21^{waf}$ and MMP9, was investigated in human bronchial epithelial cells by using real-time quantitative PCR (Figure 7). Incubation of BEAS-2B cells for 24 h in the presence of 0.5 μg/ml rDer p1 significantly up-regulated the mRNA expression of $p21^{waf}$ and MMP9 (1.35 ± 0.08 and 1.59 ± 0.12). In contrast, rDer p1 failed to augment the expression of TGF-β1 and EGFR. BEAS-2B cells displayed up-regulation of MMP9 expression when co-cultured with hEOS or differentiated EOS/HL-60 cells (3.75 ± 0.75 and 3.63 ± 0.09, respectively). This was not the result, however, for TGF-β1, EGFR, or $p21^{waf}$.

Additionally, rDer p1-stimulated hEOS or EOS/HL-60 cells influenced BEAS-2B mRNA expression of TGF-β1 (1.43 ± 0.01 and 1.42 ± 0.10, respectively), $p21^{waf}$ (1.33 ± 0.18 and 1.38 ± 0.15, respectively) and MMP9 (5.75 ± 1.77 and 4.24 ± 0.06, respectively). BEAS-2B cells treated with the conditioned medium of stimulated EOS/HL-60 cells evidenced an induced expression of TGF-β1, EGFR, $p21^{waf}$, and MMP9 (2.31 ± 0.21, 2.29 ± 0.17, 1.69 ± 0.06 and 5.32 ± 0.26, respectively).

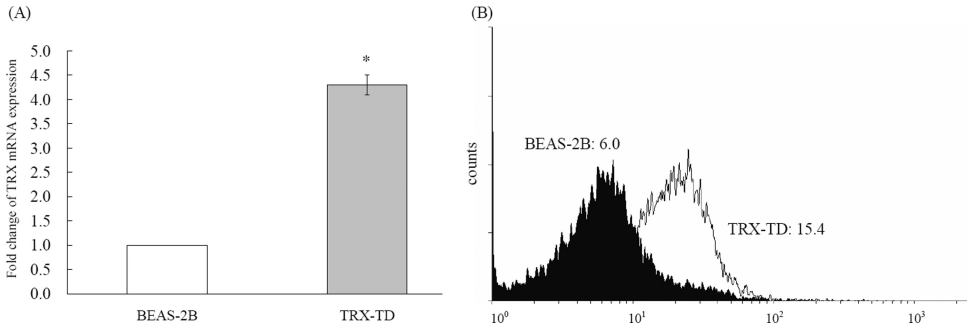

FIG. 4. Expression of inducible TRX in bronchial epithelial cells in analysis of TRX and flag-tagged TRX. TRX-TD cells were induced to over-express human TRX in the presence of doxycycline. (A) In real-time PCR analysis, TRX-TD cells expressed the higher level of total TRX mRNA than BEAS-2B cells. (B) Cytometry analysis of intracellular TRX-flag protein was stained, and the number represented as the intensity of fluorescence signal. In protein level, TRX-TD cells had the higher amount to express TRX-flag protein. It indicated the exogenous TRX was additional to TRX-TD cells over-expressing TRX. * indicates significant at $p < 0.05$ compared with BEAS-2B cells. TRX-TD: doxycycline-induced BEAS-2B Tet-on pTRE2hyg-TRX-3flag cells.

Thioredoxin Over-Expression Modulates the Inference of Remodeling Factors in Human Bronchial Epithelial Cells upon Interaction with rDer p1 and Eosinophils

That over-expression of TRX modulated gene expression of remodeling factors was shown in Figure 8. The basal Ct level of TGF-β1, EGFR, and p21^{waf} transcripts were similar in BEAS-2B and TRX-TD cells (20.9 $\pm$ 0.7 and 21.3 $\pm$ 0.4, data not shown). Otherwise, a higher basal level of MMP9 Ct was detected in TRX-TD cells compared to that in BEAS-2B cells (29.2 $\pm$ 0.3 and 31.1 $\pm$ 0.5, respectively).

The rDer p1-stimulated hEOS or EOS/HL-60 cells also influenced BEAS-2B cell mRNA expression of TGF-β1 (1.43 $\pm$ 0.01 and 1.42 $\pm$ 0.10, respectively; Figure 8A), p21^{waf} (1.33 $\pm$ 0.18 and 1.38 $\pm$ 0.15, respectively; Figure 8C), and MMP9 (5.75 $\pm$ 1.77 and 4.24 $\pm$ 0.06, respectively; Figure 8D). BEAS-2B cells treated with the conditioned medium from stimulated EOS/HL-60 cells displayed enhanced expression of TGF-β1, EGFR, p21^{waf}, and MMP9 (2.31 $\pm$ 0.21, 2.29 $\pm$ 0.17, 1.69 $\pm$ 0.06, and 5.32 $\pm$ 0.26, respectively). The transcript levels of TGF-β1 and EGFR mRNA were suppressed in TRX-TD cells treated with the medium from rDer p1-stimulated EOS/HL-60 cells (0.58 $\pm$ 0.19 and 0.35 $\pm$ 0.04, respectively) (Figures 8A and 8B).

TRX-TD cells co-cultured with hEOS or EOS/HL-60 cells in the presence of rDer p1 evidenced reduced p21^{waf} mRNA expression (1.07 $\pm$ 0.04 or 0.88 $\pm$ 0.10, respectively) compared with that by BEAS-2B cells (1.33 $\pm$ 0.18 or 1.38 $\pm$ 0.15, respectively; Figure 8C). TRX-TD cells treated with the conditioned medium of stimulated EOS/HL-60 cells displayed decreased transcript levels of EGFR and p21^{waf} (0.73 $\pm$ 0.06 and 1.24 $\pm$ 0.02, respectively) as compared to levels in the BEAS-2B cells (2.29 $\pm$ 0.17 and 1.69 $\pm$ 0.06, respectively) (Figures 8B and 8C). In contrast, treatment with the conditioned medium induced TRX-TD cells to express enhanced levels of the transcript of MMP9 (7.24 $\pm$ 0.11) as compared to the up-regulation seen with the BEAS-2B cells (5.32 $\pm$ 0.26) (Figure 8D). The amounts of MMP9 protein that were expressed by BEAS-2B and TRX cells upon interaction with rDer p1-stimulated eosinophils was shown in Figure 8E. Treatment with the conditioned medium from the stimulated EOS/HL-60 cells also induced the expression of MMP9. Induction of MMP9 protein was suppressed by adding TGF-β1 Ab.

DISCUSSION

Airway epithelial cells exposed to allergens release chemokines to recruit inflammatory cells such as eosinophils and neutrophils. These inflammatory cells would accumulate in the lung and induce sloughed airway epithelial cells to express remodeling mediators, such as growth factors, cyclin/cyclin-dependent kinase inhibitor and matrix metalloproteinase, resulting in airway remodeling in the epithelium. This study investigated ROS generation and apoptosis in human bronchial epithelial cells during interaction with HDM and activated eosinophils. Since rDer p1-simulated eosinophils caused intracellular redox changes, it also incurred oxidative stress and apoptosis in human bronchial epithelial cells. The redox protein TRX prevented bronchial epithelial cells from undergoing apoptosis and attenuated the expression of remodeling factors to resist any onset/progression of airway remodeling.

Previous studies determined that rDer p1 activates bronchial epithelial BEAS-2B cells to release IL-6 (King et al., 1998), and allergic asthmatic patients have elevated IL-6 at the local inflammatory site (Wong et al., 2001). Stimulation by rDer p1-activated human eosinophils causes BEAS-2B cells to release IL-6, which would result in increased inflammation in the lungs (Wong et al., 2006). Yoshida et al. (1999) demonstrated that TNFα induces IL-6 secretion from synovial fibroblasts by augmenting the NF-κB activation pathway. Experimental results in this study indicated that BEAS-2B cells co-cultured with rDer p1-activated eosinophils potentially promoted IL-6 release in

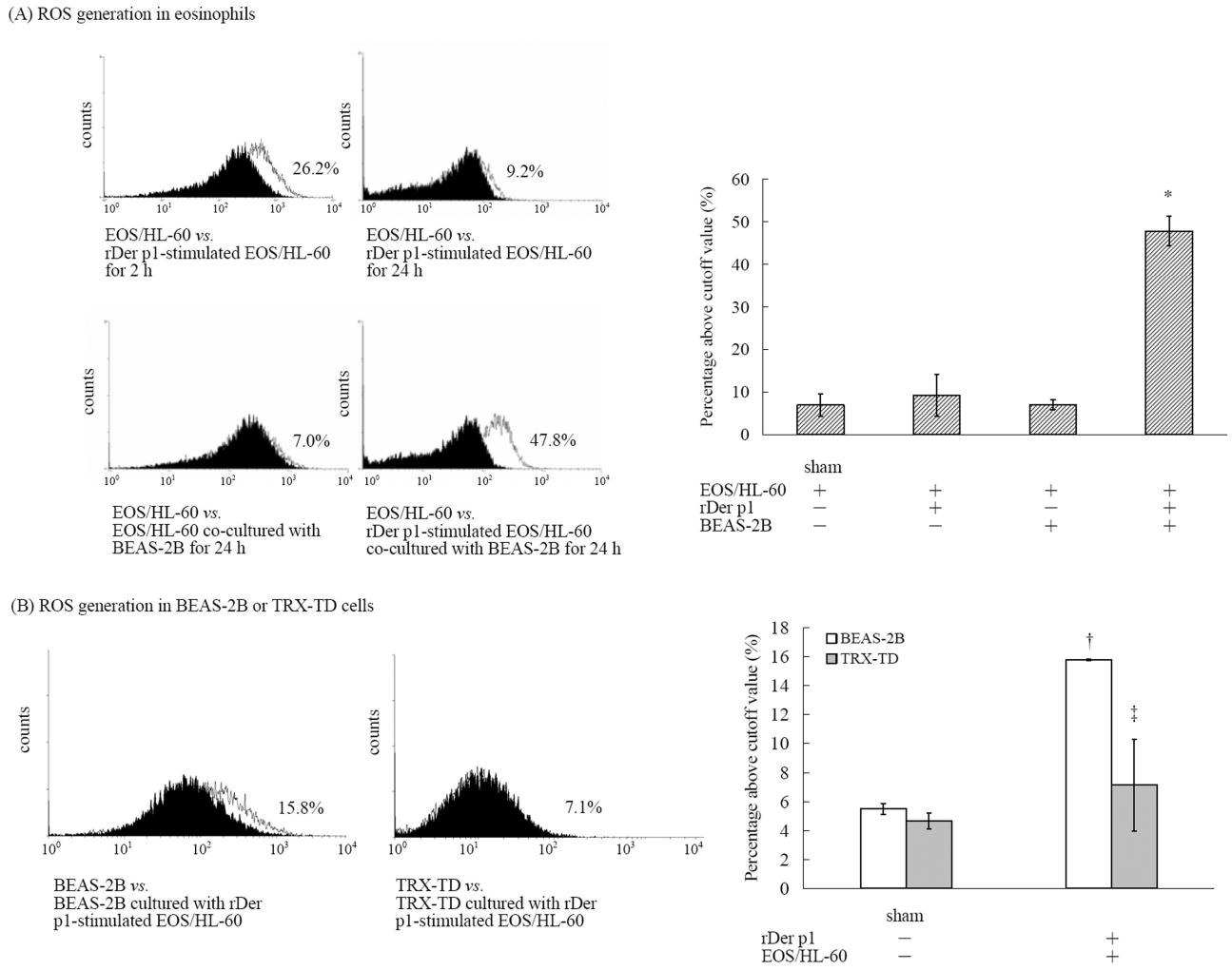

FIG. 5. ROS generation in human eosinophils, BEAS-2B and TRX-TD cells. Differentiated HL-60/clone 15 eosinophils stimulated with 0.5 μg/ml rDer p1 for 2 or 24 h. Confluent BEAS-2B cells and TRX-TD cells independently co-cultured with rDer p1-stimulated EOS/HL-60 cells for other 24 h. ROS generation was determined by CM-H_2DCFDA and FACS analysis. (A) Differentiated EOS/HL-60 cells were induced to generate ROS by 2-h rDer p1 stimulation, and declined after 24 h. When cultured with BEAS-2B cells, the rDer p1-stimulated eosinophils obviously generated ROS. (B) ROS generation was induced in BEAS-2B cells but suppressed in TRX-TD cells. * indicates significant at $p < 0.05$ compared with rDer p1-stimulated EOS/HL-60 cells in RPMI medium, † at $p < 0.05$ compared with sham BEAS-2B cells in RPMI medium, and ‡ at $p < 0.05$ compared with BEAS-2B cells cultured with rDer p1-stimulated EOS/HL-60 cells.

order to regulate the inflammatory response (Figure 2). An increased TNFα level was detected in the supernatant of EOS/HL-60 cells after rDer p1 activation upon interaction with BEAS-2B cells (Figure 3). These results meant that EOS/HL-60 cells could represent primary blood eosinophils that interact with bronchial epithelial cells and that were primarily responsible for TNFα secretion after rDer p1 stimulation.

Moreover, following *ex vivo* stimulation, cells recovered from bronchoalveolar lavage fluid and the blood of asthmatic subjects generate greater amounts of ROS than do cells from normal subjects; this outcome is also correlated with disease severity (Sanders et al., 1995). Cellular sources of ROS in the lung include eosinophils, neutrophils, and alveolar macrophage, as well as bronchial and alveolar epithelial cells. The oxidative stress induced by, and direct damage from, these ROS can lead to peroxidation of membrane lipids, cytoskeleton disruption, DNA damage, and apoptosis (Rahman et al., 2006). Oxidative injury resulting from eosinophils can be substantial, as these cells possess a greater capacity to generate O_2^- and H_2O_2 than neutrophils (Slungaard et al., 1990). It has been shown that mite protease *Dermatophagoides farinae* 1 markedly induces superoxide anion production and degranulation in human eosinophils (Miike and Kita, 2003). In the study reported here, rDer p1 induced differentiated EOS/HL-60 cells to generate ROS within 2 h; this effect was sustained for 24 h while the cells were co-cultured with bronchial epithelial cells. In contrast, TRX-TD cells that

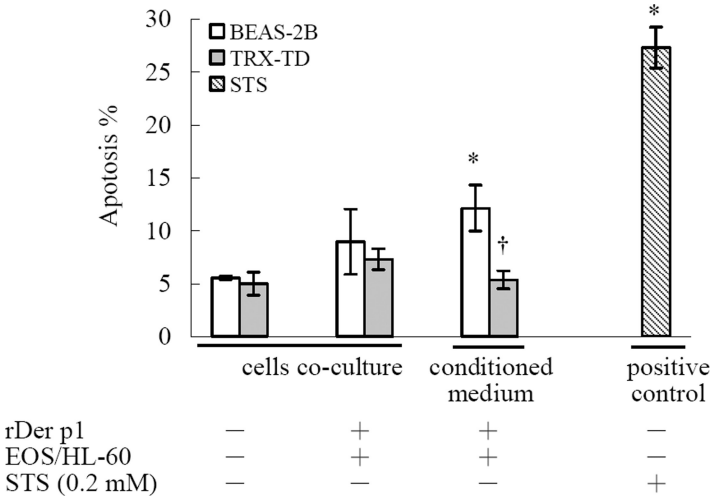

FIG. 6. Apoptosis in human BEAS-2B and TRX-TD cells. Differentiated EOS/HL-60 cells were previously treated 0.5 μg/ml rDer p1 for 24 h. Confluent BEAS-2B cells and TRX-TD cells independently co-cultured with rDer p1-stimulated EOS/HL-60 cells or conditioned medium of stimulated EOS/HL-60 cells for 24 h. The cells were stained with Annexin-V-FITC and analyzed for apoptosis by FACS detector. Intact cells and apoptotic cells, separately, located in the lower left and right quadrants. Human TRX-TD cells over-expressing TRX suppressed apoptosis while co-cultured with rDer p1-stimulated EOS/HL-60 cells. STS: staurosporine causes mitochondria damage as positive control of apoptosis of BEAS-2B cells. * indicates significant at $p < 0.05$ compared with sham BEAS-2B cells in RPMI medium, and † at $p < 0.05$ compared with BEAS-2B cells cultured in the conditioned medium of rDer p1-stimulated EOS/HL-60 cells.

over-expressed TRX were seen to suppress ROS production during interaction of eosinophils-bronchial epithelial cells and rDer p1.

Eosinophils cause apoptosis and necrosis of bronchial epithelial cells (Trautmann et al., 2002). Activated eosinophils can induce airway cell damage by releasing ROS (Giembycz and Lindsay, 1999; Coward et al., 2004), TNFα (Slungaard et al., 1990; Dworski, 2002; Andreadis et al., 2003; Pascual and Peters, 2005), and granular proteins (Walsh, 2001; Fan et al., 2007). TNF-α transcriptionally regulates HDM-induced gene expression of airway epithelial cells at earlier time point (Vroling et al., 2007). The current study showed that BEAS-2B cells underwent apoptosis when treated with conditioned medium from rDer p1-activated eosinophils. An increased TNFα level was also found in the medium of EOS/HL-60 cells after rDer p1 activation (upon interaction with BEAS-2B cells). This indicated that eosinophils secreted mediators relevant to induction of apoptosis in bronchial epithelial cells. Again, in contrast, TRX-TD cells over-expressing TRX were seen to be able to decrease the degree of inducible apoptosis.

Airway remodeling occurs during the initiation of tissue repair in response to continuous allergic inflammation in the airway. It is unclear why eosinophils predominate in allergic inflammatory foci, as opposed to other innate immune cells such as monocytes and neutrophils. Part of the answer may be that eosinophils appear to have a particular affinity for inhalant allergens (Svensson et al., 2004). HDM allergens are Th2 cell adjuvants, and proteolytically degrade tight junctions in airway epithelium and cause release of pro-inflammatory cytokines from airway epithelial cells (Chapman et al., 2007). A currently accepted model of airway allergic diseases caused by chronic inflammation is directed by Th2 cells reacting to inhaled allergens and antigens (Wardlaw et al., 2002). Eosinophils are Th2 effector cells that cause tissue damage/dysfunction by releasing toxic granule proteins and lipid mediators (Rothenberg and Hogan, 2006). Repeated instillation of HDM into airways induces Th2-dependent airway hyperresponsiveness and eosinophilia; increasing percentage of activated eosinophils is more than neutrophils (Wakahara et al., 2008). Pegorier et al. (2006) reported that eosinophil-derived granular proteins, major basic proteins, or peroxidase, directly up-regulate transcripts of remodeling factors, including endothelin-1, TGF-β1, platelet-derived growth factor-β, EGFR, MMP9, fibronectin, and tenascin, in human bronchial epithelial cells. Most gene expression of remodeling factors (including TGF-β1, EGFR and $p21^{waf}$) was seen to be increased in the airways of asthmatics, and the extent of expression frequently correlates with disease severity (Sanders et al., 1995; Minshall et al., 1997; Redington et al., 1997; Vignola et al., 1997; Hoshino et al., 1998; Trautmann et al., 2002; Hamilton et al., 2003). The quantitative PCR results in the current study indicated that rDer p1-stimulated eosinophils induced TGF-β1 trascripts in BEAS-2B cells (Figure 7). Additionally, TGF-β1 could induce cell apoptosis and precede inflammatory response (Blobe et al., 2000). The results here suggested that rDer p1-stimulated eosinophils induced TGF-β1 expression in BEAS-2B cells and this, in turn, resulted in apoptosis.

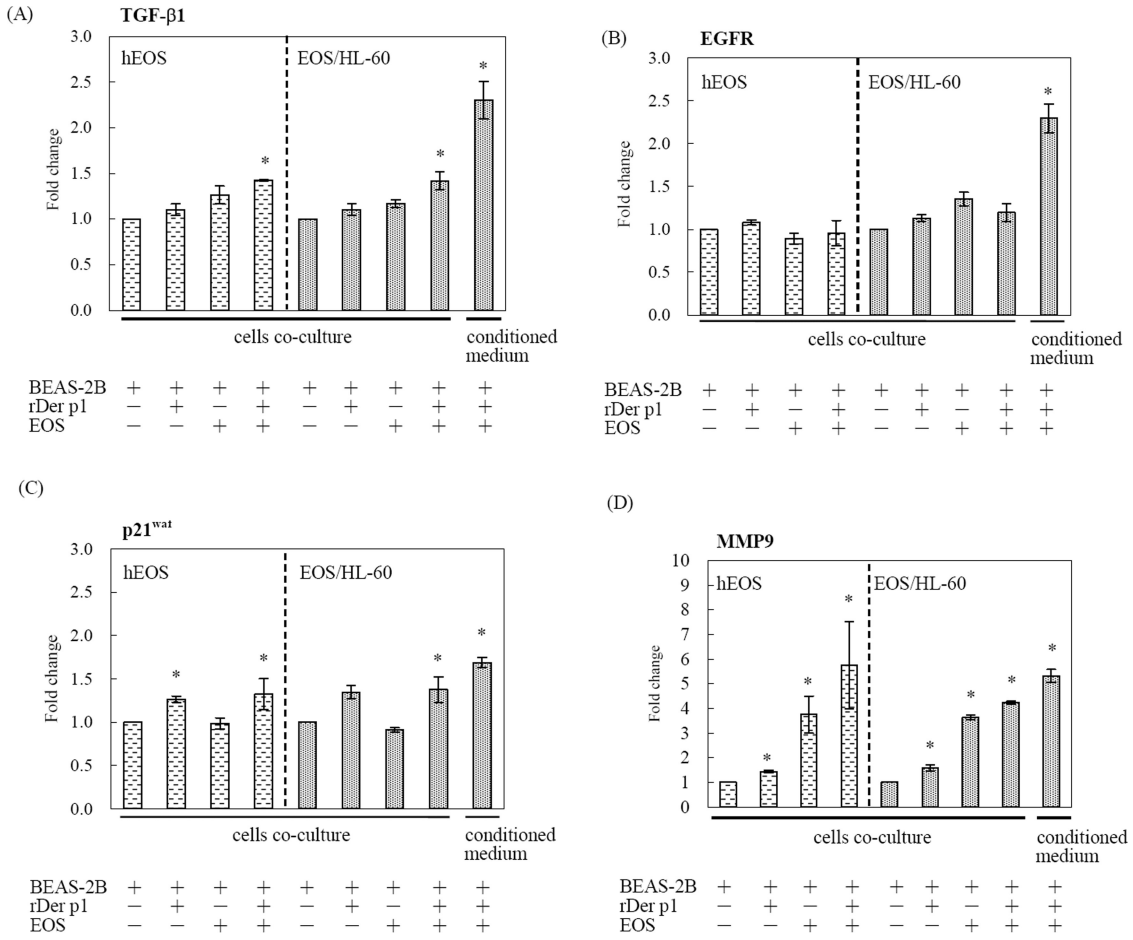

FIG. 7. Gene expression of remodeling factors in human bronchial epithelial cells upon interaction with rDer p1 and eosinophils. The mRNA expression of airway remodeling factors (A) TGF-β1, (B) EGFR, (C) p21^{waf} and (D) MMP9 was performed by a real-time quantitative PCR. Incubation of BEAS-2B cells with rDer p1 significantly up-regulated mRNA expression of p21^{waf} and MMP9. Regardless of rDer p1 stimulation or not, BEAS-2B cells had the up-regulation of MMP9 expression when co-cultured with hEOS or differentiated EOS/HL-60 cells. BEAS-2B cells treated with the conditioned medium of stimulated EOS/HL-60 cells induced the expression of TGF-β1, p21^{waf}, EGFR and MMP9. Results were expressed as the ratio of each transcript relative to geometrical average of mRNA expression of β-actin, mean $\pm$ SD for two independent experiments. * indicates significant at $p < 0.05$ compared with BEAS-2B in RPMI medium.

TGF-β1 has a further crucial role in the signal network of cell growth and differentiation (Massagué, 2002). TGF-β1 can activate EGFR for anti-apoptosis and cell survival (Murillo et al., 2005). EGFR may contribute directly to these pro-inflammatory responses and elicit IL-8 release from bronchial epithelial cells (Hamilton et al., 2003). Fedorov et al. (2005) demonstrated that the thickness of the lamina reticularis is significantly correlated with epithelial EGFR expression. EGFR impairs apoptosis and induces cell proliferation through increased p21^{waf} expression (Sheng et al., 2006). p21^{waf} blocks cell apoptosis by interacting with pro-apoptotic molecules such as apoptosis signal-regulating kinase (ASK) 1, procaspase-3, and procaspase-8 (Gartel and Tyner, 2002). IL-6-type cytokines inhibit TGF-β1-induced apoptosis, and cause the p21^{waf} promoter to express p21^{waf} to protect cells from apoptosis (Bellido et al., 1998; Gartel and Tyner, 2002). According to the results of the current study, treatment with conditioned medium from rDer p1-stimulated EOS/HL-60 cells induced the BEAS-2B cells to express TGF-β1, EGFR, p21^{waf}, and MMP9 (Figure 7). This result suggested that TGF-β1 activated EGFR and p21^{waf} to mediate cell repair and proliferation after the stimulation. It has been demonstrated that TGF-β1 transgenic mice manifest impressive bronchoalveolar and tissue inflammation, fibrosis, and pulmonary alveolar remodeling (Yamasaki et al., 2008). Based on the results here, it can be assumed that eosinophils can activate bronchial epithelial cells to synthesize growth factors and extracellular matrix underlying airway remodeling.

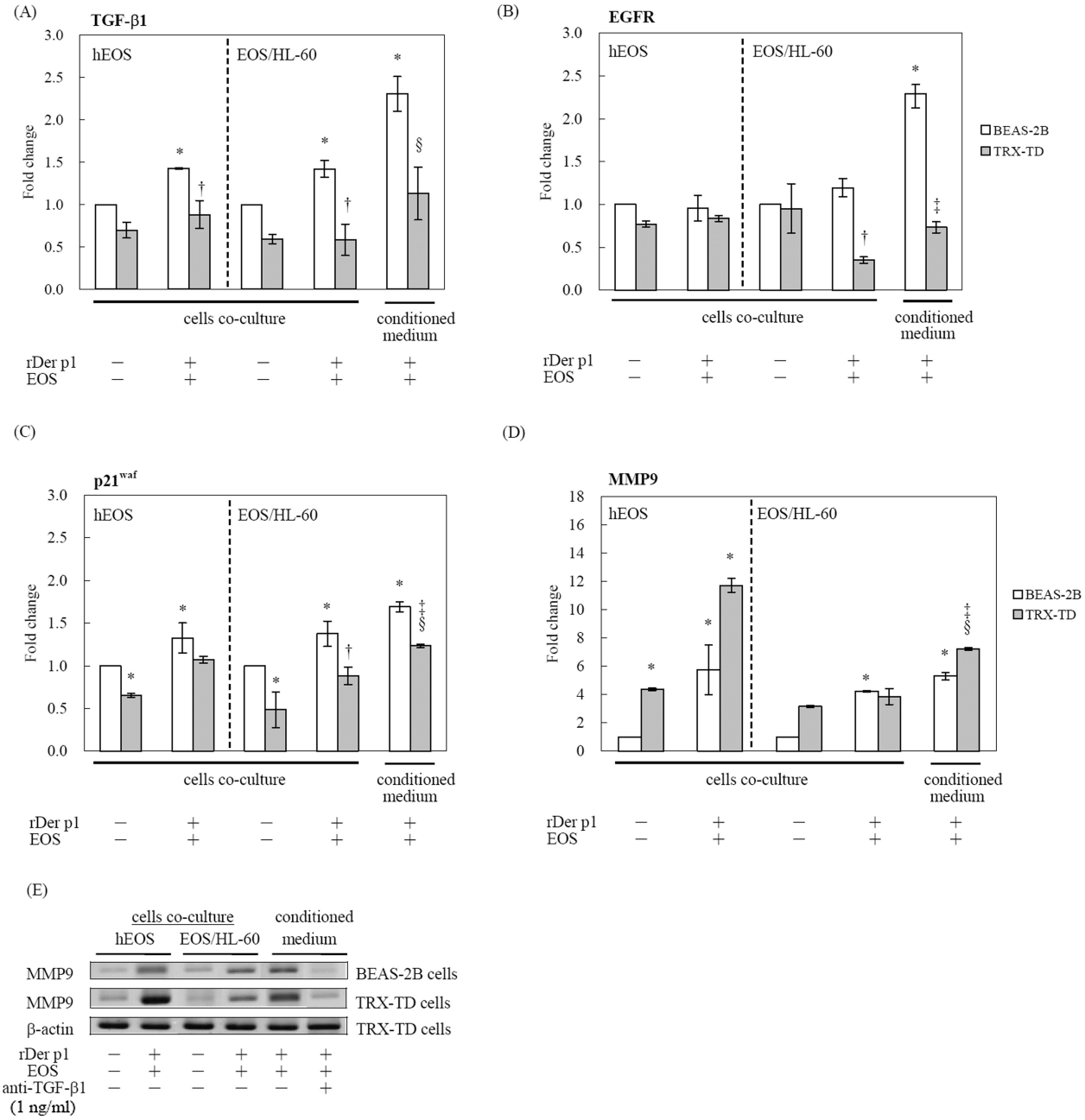

FIG. 8. TRX over-expression modulated the airway remodeling factors in human bronchial epithelial cells upon interaction with rDer p1 and eosinophils. TRX-TD cells co-cultured with eosinophils in the presence of rDer p1 reduced the expression of (A) TGF-β1, (B) EGFR, and (C) p21^{waf}, but activated (D) MMP9 expression. TRX-TD cells treated with the conditioned medium induced the up-regulation of TGF-β1, p21^{waf} and MMP9 expression. Results were expressed as the ratio of each transcript relative to the geometrical average of mRNA expression of β-actin, mean $\pm$ SD for two independent experiments. (E) MMP9 protein was determined by immunoblot individually from BEAS-2B and TRX-TD cells with treatments. MMP9 protein was activated by rDer p1-stimulated eosinophils. TGF-β1 Ab can neutralize the eosinophil-derived mediators in the conditioned medium, and suppressed the activation of MMP9 protein. * indicates significant at $p < 0.05$ compared with BEAS-2B in RPMI medium, † at $p < 0.05$ compared with BEAS-2B cultured with rDer p1-stimulated ESO/HL-60, ‡ at $p < 0.05$ compared with BEAS-2B cultured with conditioned medium of rDer p1-stimulated ESO/HL-60, and §at $p < 0.05$ compared with TRX-TD in RPMI medium.

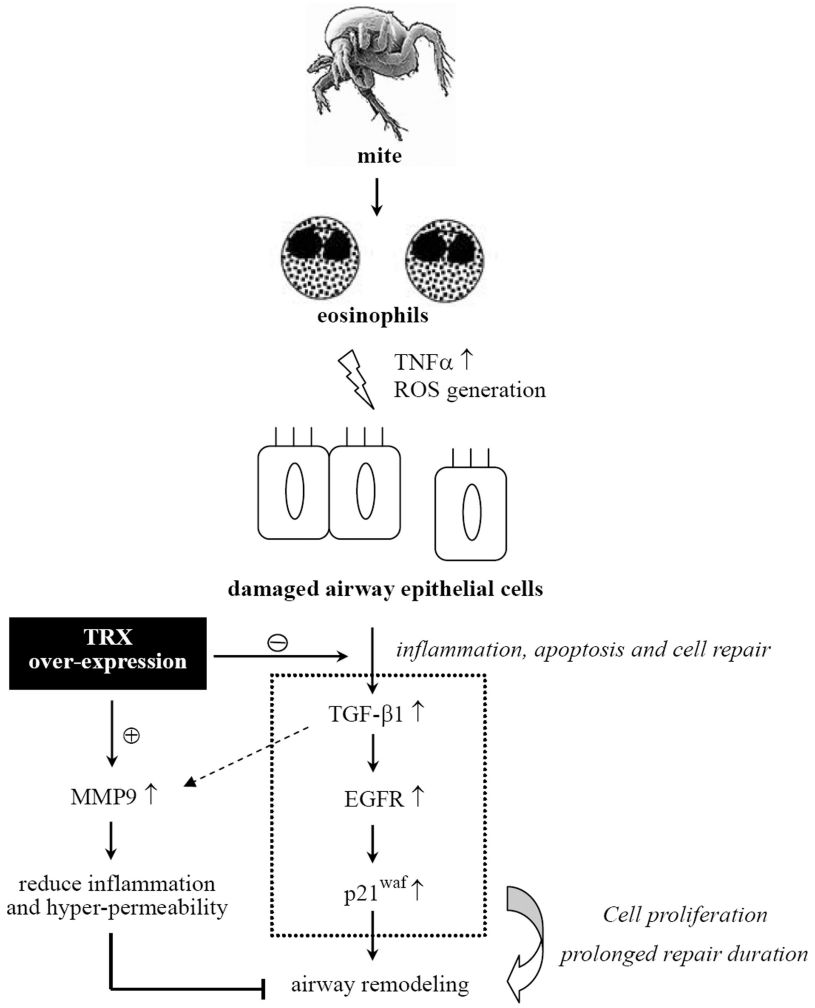

FIG. 9. An illustration of TRX mediating remodeling factors in bronchial epithelial cells upon mite-stimulated eosinophils interaction. Sloughed epithelial cells expressing TGF-β1, EGFR and p21^{waf} contributed to apoptosis and were implicate to airway remodeling due to irregular cell proliferation and prolonged repair duration. Transgenic TRX attenuated the expression of TGF-β1, EGFR and p21^{waf}and up-regulated MMP9 for the reduction of inflammation and hyper-permeability. It suggested TRX can mediate the attack of mite-stimulated eosinophils to retard apoptosis and the progression of airway remodeling in bronchial epithelial cells.

Damaged epithelial cells may prolong the period of epithelial repair and contribute to airway remodeling. Up-regulation of MMP9 is thought to modulate airway remodeling (Ohbayashi and Shimokata, 2005) and induces digestion of basement membranes. MMP9 has been shown to have a protective capability during ozone-induced lung neutrophilic inflammation and hyper-permeability (Yoon et al., 2007). Enhanced activation of MMP9 also promotes airway epithelial wound repair (Bove et al., 2007). Additionally, TGF-β1 stimulates the expression of MMP9 for cell migration (Seomun et al., 2008). In this study, activation of MMP9 protein was suppressed by treatment with an anti-TGF-β1 Ab. In addition, a presence of TGF-β1 appeared to neutralize eosinophil-derived mediators in the conditioned medium, and attenuated the generation of MMP9 protein. These outcomes suggested that eosinophil-derived mediators induced MMP9 protein expression by BEAS-2B cells through effects upon TGF-β1 secretion.

TRX has stress inducible characteristics to prevent cell injury and apoptosis (Kaimul Ahsan et al., 2005; Sato et al., 2006). TRX is involved in cell growth and differentiation (Arner and Holmgren, 2000), and regulation of tumor suppressor p53 to transactivate p21^{waf} for cell cycle progression, DNA repair and apoptosis (Ueno et al., 1999). Genetic suppression or inhibition of TRX results in increased ROS and apoptosis (Hansen et al., 2006). TRX transgenic mice decreased alveolar damage from hyperoxia-induced apoptosis (Yamada et al., 2007). TRX prevents airway remodeling and eosinophilic infiltration into the lungs of chronically-OVA-exposed BALB/c:human TRX1 transgenic mice (Imaoka et al., 2007). However, it is still not known if, nor is a mechanism yet defined as to how, TRX

mediates remodeling factor in airway epithelial cells exposed to house dust mite. In this study, it was shown that TRX over-expression reduced the transcript levels of TGF-β1 to prevent apoptosis during the co-culture of TRX-TD cells with eosinophils in the presence of rDer p1 (Figure 8). Additionally, TRX inhibited irregular cell proliferation and repair by down-regulating EGFR and $p21^{waf}$ expression in TRX-TD cells during the interaction with the rDer p1-stimulated eosinophils. This finding suggested that TRX could reduce gene expression of TGF-β1, EGFR, and $p21^{waf}$ to eliminate the influence of any irregular cell proliferation of extracellular matrices on airway epithelial cells. In contrast, TRX over-expression enhanced MMP9 transcription in TRX-TD cells. Up-regulated MMP9 expression is likely to have a protective role for bronchial epithelial cells during an inflammatory response. The results in the study here showed that TRX over-expressing bronchial epithelial cells activated MMP9 and attenuated TGF-β1 expression, thereby preventing cell proliferation and modification/turnover of matrix composition that can contribute to airway remodeling. Figure 9 presents an illustration of how TRX might mediate remodeling factors after the interaction between HDM-stimulated eosinophils and human bronchial epithelial cells.

House dust mite is a bronchial epithelial allergen that causes allergic disorders. This study cultured human bronchial epithelial cells in the presence of rDer p1 and/or eosinophils to mimic the *in vivo* interactions between bronchial epithelial cells and eosinophils during allergic inflammation. The protease activity of rDer p1 induced eosinophils to generate ROS and TNFα, which resulted in oxidative stress and apoptosis in bronchial epithelial cells. The sloughed epithelial cells generated relevant factors that were implicated in bronchial epithelial remodeling. Moreover, this study investigated how the redox protein TRX might reduce the oxidative stress in human bronchial epithelial cells caused by rDer p1-stimulated eosinophils. TRX mediated gene expression of remodeling factors that potentially contributed to extracellular matrix modification through TGF-β1 attenuation and MMP9 activation in human bronchial epithelial cells. These results suggested that TRX over-expression in human bronchial epithelial cells could mediate oxidative damage and gene expression of remodeling factors from inflammation in response to an allergen-immune reaction. Regarding its regulatory capability, TRX has the potential to be a therapeutic agent for eosinophil-mediated airway illnesses.

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