

Tissue Transglutaminase Can Be Involved in Airway Inflammation of Toluene Diisocyanate-Induced Occupational Asthma

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Abstract

Background This study was conducted to evaluate whether tissue transglutaminase (tTG) may be involved in airway inflammation of toluene diisocyanate-induced occupational asthma (TDI-OA).

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Methods We enrolled 93 patients with TDI-OA, 177 asymptomatic exposed subjects, 43 patients with allergic asthma, and 70 unexposed normal controls. The prevalence of serum immunoglobulin G (IgG) to tTG in the TDI-OA group (20.2%) was significantly higher than that in the three other groups ($P < 0.001$).

Results TDI-OA patients with serum IgG to tTG had significantly lower methacholine PC₂₀ values ($P < 0.02$) and significantly higher prevalence of specific immunoglobulin E to vapor type TDI-human serum albumin conjugate ($P < 0.01$; $r^2 = 0.411$, $P < 0.05$). TDI exposure could increase tTG activity via reactive oxygen species (ROS) production, which was found to cross-link with cytokeratin 19 on immunoblot analysis.

Conclusion Therefore, TDI exposure may activate tTG via ROS-mediated mechanism in the airway epithelium leading to persistent airway inflammation in TDI-OA patients.

Keywords Occupational asthma · specific immunoglobulin E · toluene diisocyanate · transglutaminase

Introduction

Toluene diisocyanate (TDI) exposure is the most common cause of occupational asthma (OA) worldwide [1, 2]; in Korea, its prevalence is 2–13% in exposed workers [3–5]. Although many researchers have investigated its pathogenic mechanism, it is still not fully understood. However, previous studies detected a significant increase in the level of serum-specific immunoglobulin E (IgE) after the application of a vapor type TDI-human serum albumin (HSA) conjugate, indicating that IgE may play a role in TDI-induced OA (TDI-OA) [6]. An immunohisto-

chemical study of the airway mucosa of TDI-OA patients showed inflammatory cell infiltration, including activated neutrophils, with persistent epithelial injury [7, 8]. Cytokeratin (CK) is the major structural protein of the bronchial epithelial cells, and as an activated immunologic mechanism, increased expression of CK19 was noted after exposure to TDI. The prevalence of serum-specific immunoglobulin G (IgG) to CK19 was significantly higher than that of asymptomatic exposed workers or normal controls [9]. Tissue transglutaminase (tTG) is a calcium-dependent enzyme responsible for the post-translational modification of tissue proteins or polypeptides, which is activated in epithelial cells and is a self-antigen for several autoimmune diseases, including celiac disease and dermatitis herpetiformis [10–13].

In order to understand the pathogenic mechanisms of TDI-OA, especially whether there is an autoimmune mechanism and whether CK is released to generate autoantibody and to evaluate the relationship between tTG and IgG to CK19, we analyzed the prevalence of serum-specific IgG to tTG and evaluated the relationship with serum-specific IgE and IgG antibodies to TDI–HSA conjugate in a larger cohort of TDI-OA patients.

Materials and Methods

Subjects

All subjects were recruited from four major medical centers in Korea, and they had been working in isocyanate-exposed workplaces, such as spray painting or varnishing of furniture or musical instrument. For identification of TDI-OA patients, first, we did questionnaire survey to screen for the presence of work-related respiratory symptoms. Then, we did methacholine and specific inhalation challenge tests for workers complaining of work-related respiratory symptoms. Based on these steps, 93 subjects with TDI-OA were enrolled, and their diagnosis was confirmed by positive responses to methacholine bronchial challenge and TDI-specific bronchial challenge tests. The control subjects included 177 asymptomatic exposed subjects (AECs) in similar TDI-exposed environments, but having no work-related respiratory symptoms. Seventy normal healthy controls (NC) and 43 allergic asthma subjects (AA) were recruited from those who had never been exposed to TDI by personal history.

Atopy status was determined by a positive skin prick test to at least one common inhalant allergen, such as house dust mites, tree and pollen mixtures, mugwort, ragweed pollens, or *Alternaria* (Bencard, Bradford, UK). All subjects underwent an interview and chest radiography. To

confirm TDI-OA, we subjected patients to lung function measurements and inhalation tests with methacholine and TDI described in the prevalence studies [5, 8]. Sera were collected from study subjects during the time of isocyanate-specific inhalation test for TDI-OA patients. The sera from AEC and healthy control subjects were collected at initial examination. All participants provided their informed consent, which was regulated by the institutional review board of Ajou University Medical Center, Suwon, South Korea.

Bronchoprovocation Tests with Methacholine and TDI

The methacholine challenge was performed on subjects suspected of having TDI-OA or AA as previously described [6, 14]. The methacholine PC₂₀ value, or the concentration of methacholine needed to produce a 20% decrease in FEV₁, was determined by interpolation from a dose–response curve. TDI-specific inhalation challenge tests were performed within 1 month after last exposure of isocyanate as previously described [6, 14].

Specific IgG and IgE Antibody to TDI–Human Serum Albumin Conjugate by Enzyme-Linked Immunosorbent Assay

Serum-specific IgE and IgG levels were detected using enzyme-linked immunosorbent assays (ELISAs) as previously described [6]. Vapor-type TDI–HSA conjugate was kindly provided by Dr. Adam Wisniewski of Yale University, USA. At first, 1 µg of TDI–HSA or mock conjugate was dissolved in normal saline and used to coat ELISA plates (Corning, New York, NY, USA). After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T), plates were blocked with blocking buffer [PBS containing 5% bovine serum albumin (BSA) and 0.05% Tween 20]. Diluted sera (50 µL) from patients and control subjects were incubated for 1 h at 25°C in both TDI–HSA and mock HSA-coated wells. After washing, alkaline phosphatase-conjugated anti-IgG (1:2,000, 100 µL; Sigma, St. Louis, MO, USA) or biotinylated anti-IgE antibody (1:1,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), followed by alkaline phosphatase-conjugated streptavidin (1:500, Sigma) was added to each well, and then, 100 µL of a substrate solution (Sigma) was added. Absorbance values were read using an ELISA reader at wavelengths of 405/450 nm. To quantify antibody-binding specificity to TDI, we subtracted the optical density of the mock HSA-conjugated wells from that of the TDI–HSA-coated wells. Positive cutoff values were determined by the mean + threefold standard deviation of absorbance values from tests on unexposed healthy controls.

Serum IgG to tTG by ELISA

Serum IgG to tTG level was measured using ELISA kits (Orgentec, Mainz, Germany) following the instruction manual. Positive cutoff value was 10 U/mL from the instruction.

Epithelial Cell Culture and tTG Activity Assay

A549 cells, a human airway epithelial cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). They were grown in RPMI-1640 with 10% fetal bovine serum and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. In situ tTG activity was determined via fluorescence microscopy using a previously described method with modifications [15]. Briefly, 2×10^4 A549 cells seeded in 24-well plates were incubated with 1 mM 5-(biotinamido) pentylamine (Pierce, Rockford, IL, USA) and subsequently treated with TDI-HSA. After washing, they were fixed with PBS containing 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 for 5 min at room temperature. After washing, the cells were blocked with PBS containing 5% BSA for 30 min. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:200; Millipore, Billerica, MA, USA) in PBS for 1 h at room temperature. The stained cells were observed under a fluorescence microscope (Carl Zeiss, Jena, Germany). More than 30 cells per field were randomly selected from each of five fields in three separate experiments, and fluorescent intensity was determined by processing FITC images at the cellular level. tTG activity was determined by comparing the fluorescent intensity of treated cells to that of unstimulated control cells and presented as A.U. (arbitrary unit) calculated by the fluorescent intensity of the sample divided by that of control.

Intracellular Reactive Oxygen Species Measurement

A549 cells (2×10^5) seeded in 60-mm culture dishes were incubated with 5 μ M 2',7'-dichlorodihydrofluorescein diacetate (Sigma) for 30 min at 37°C. After washing, the cells were incubated with each concentration of the TDI-HSA conjugate. The cells were then washed with PBS and detached by trypsination. After washing, 1×10^4 cells per sample were immediately analyzed using a FACS Vantage (Becton Dickinson). Data were analyzed using WinMDI 2.8 (Scripps Research Institute, La Jolla, CA, USA) and presented as A.U. calculated by the mean fluorescent intensity of the sample divided by that of control.

Immunoblot Analysis

A549 cells (2×10^5) seeded in 60-mm culture dishes were incubated in the presence or absence of TDI-HSA for 24 h. The cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 2 mM ethylenediamine tetraacetic acid (EDTA)) containing a mixture of protease inhibitors (Sigma). After homogenization on ice for 30 min and centrifugation at 13,000 rpm at 4°C, the supernatants were used as the soluble fraction. The pellets were again resolved by 8 M urea in PBS, and each fraction was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membranes were blocked in blocking solution (PBS containing 5% nonfat dried milk) and then probed with anti-tTG (Lab Vision, Fremont, CA, USA), anti-CK19 (Santa Cruz Technology, Santa Cruz, CA, USA), or anti-tubulin (Sigma) for 1 h at room temperature. After washing in PBS-T, the membranes were incubated with secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. After washing, the membranes were developed using an ECL solution (Pierce) and exposed to Kodak X-ray film.

In Vitro Cross-linking Reaction Assay

The in vitro cross-linking reaction assay was performed as previously described [16]. Briefly, the reaction was performed at 37°C for 2 h in a buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 2 mM leupeptin, and 4 μ M recombinant CK19 (Progen Biotechnik GmbH, Heidelberg, Germany). Depending on the reaction, purified guinea pig liver tTG (Sigma), calcium chloride (2 mM), and/or EDTA (2 mM) were added. The reaction was quenched by the addition of SDS sample buffer, and the products were subjected to SDS-PAGE, followed by Western blot analysis.

Statistical Analysis

Statistical analyses were performed using the software SPSS, Version 12.0 (Chicago, IL, USA). To compare clinical parameters of four study groups, one way ANOVA and χ^2 test were used, and continuous values were adjusted for age and sex using analysis of covariance (ANCOVA). For subgroup analysis, Student's *t* test and χ^2 test were used to compare demographic data, and the obtained values were adjusted for age and sex. Pearson's correlation coefficient was obtained to evaluate the correlation between IgE to TDI-HSA and IgG to tTG. Mann-Whitney *U* test was used to evaluate tTG and reactive oxygen species (ROS) levels derived from cell cultures. *P* < 0.05 was regarded as significant.

Table 1 Clinical and Immunological Data of the Study Subjects

	TDI-OA (<i>n</i> =93)	AEC (<i>n</i> =177)	AA (<i>n</i> =43)	NC (<i>n</i> =70)	<i>P</i>
Age	41.84±9.98	41.29±8.18	27.79±9.29	30.55±9.90	<0.001
Male (%)	62 (66.7)	130 (73.4)	24 (56.8)	30 (42.9)	<0.001
Exposure period (years)	6.27±3.77	11.97±7.20	NA	NA	0.01 ^a
Smoking (%)	20 (28.2)	66 (45.8)	4 (14.3)	10 (16.4)	0.539
FEV ₁ (%)	85.50±22.28	90.17±20.24	91.42±18.75	NA	0.546 ^a
PC ₂₀ (mg/mL)	8.33±15.54	NA	3.10±5.68	NA	0.381 ^a
Log total IgE (kU/L)	4.96±1.4	4.65±1.39	5.84±1.04	3.65±1.24	<0.001 ^a
sIgE to TDI-HSA (%)	32 (34.8)	9 (5.1)	0 (0)	1 (1.6)	<0.001
sIgG to TDI-HSA (%)	27 (29.7)	18 (10.2)	1 (2.4)	2 (3.2)	<0.001
sIgG to tTG (%)	19 (20.4)	1 (0.6)	0 (0)	1 (1.4)	<0.001

TDI-OA patients with toluene diisocyanate-induced occupational asthma, AEC asymptomatic exposed controls, AA patients with allergic asthma, NC unexposed healthy controls, FEV₁ forced expiratory volume in 1 s, PC₂₀ concentration of methacholine required to produce a 20% decrease in FEV₁, sIgE serum-specific IgE antibodies, TDI-HSA toluene diisocyanate-human serum albumin, sIgG serum-specific IgG antibodies, tTG tissue transglutaminase, NA not available

^a Values were adjusted for age and sex using multiple linear regression model

Results

Demographic Data

Table 1 summarizes the relevant demographic data. The subjects of TDI-OA and AEC groups had significantly older age and males were predominant. In addition, exposure duration was significantly longer in the AEC than in the TDI-OA group. The prevalence of serum-specific IgE and IgG to vapor-type TDI-HSA was 34.8% and 29.7%, respectively, in the TDI-OA patients. These values were significantly higher than those for the AEC (5.1% and 10.2%), NC (1.6% and 3.2%), and AA (0% and 2.4%) groups ($P<0.001$ for all groups).

Serum-specific IgG to tTG was present at a significantly higher proportion in TDI-OA subjects compared to the three other control groups (TDI-OA, 20.2%; AEC, 0.6%; AA, 0%; NC, 1.4%; $P_c<0.001$; Fig. 1). Table 2 summarizes our comparative data for clinical parameters related to IgG to tTG level in TDI-OA patients. Although there were no significant differences in age, atopy, total IgE, or FEV₁, females were predominant in TDI-OA subjects who had IgG to tTG ($P=0.015$), and methacholine PC₂₀ level was also significantly lower in subjects with high serum IgG to tTG ($P=0.021$). The prevalence of specific IgE, but not specific IgG, to TDI-HSA conjugate was also significantly higher in TDI-OA patients with high IgG to tTG levels (63.2% vs. 27.8%; $P=0.004$), with a significant correlation between specific IgE and IgG to tTG levels (by Pearson's coefficient, $r^2=0.411$, $P<0.05$; data not shown).

TDI-HSA Conjugate Increased tTG Activity in A549 Cells via ROS Production

To evaluate the effect of TDI-HSA conjugate on tTG activity, we performed a tTG activity assay using fluorescent microscopic measurement of pentylamine-modified proteins in A549 cells. As shown in Fig. 2, when A549 cells were treated with TDI-HSA for 3 h, a strong increase in fluorescence was observed in the cytosol and nuclear area compared to unstimulated cells (Fig. 2a). TDI-HSA also induced tTG activity in a dose-dependent manner (Fig. 2b). Furthermore, we observed changes in intracellu-

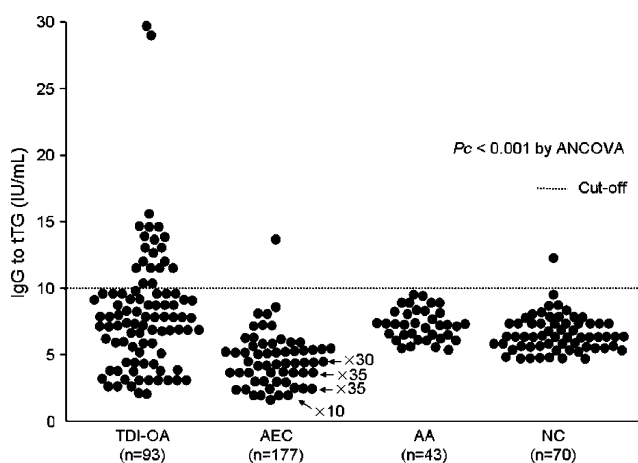


Fig. 1 Prevalence of serum IgG to tissue transglutaminase (tTG) in four study groups. Corrected P value (P_c) was obtained using ANCOVA with Bonferroni's correction to adjust age, gender, and presence of specific IgE and IgG to TDI-HSA. TDI-OA patients with toluene diisocyanate-induced occupational asthma, AEC asymptomatic exposed controls, AA patients with allergic asthma, NC unexposed healthy controls

Table II Comparison of the Clinical and Immunological Features of 93 Patients with TDI-OA According to the Presence of Serum Specific IgG to tTG

	IgG to tTG		<i>P</i>
	Presence	Absence	
Age (years) ^a	44.94±9.48	41.00±10.02	0.155
Male (%) ^b	8/19 (42.1%)	54/74 (73.0%)	0.015
Atopy ^b	7/13	26/58	0.759
Smoking ^b	6/18	14/58	0.170
Exposure period (years) ^a	8.00±2.83	6.16±3.84	0.513
FEV ₁ (%) ^a	70.98±26.51	87.15±21.43	0.092
Methacholine PC ₂₀ (mg/mL) ^a	1.22±1.47	9.28±16.33	0.021 ^c
Log total IgE (kU/L) ^a	5.07±1.25	4.94±1.48	0.693
sIgE to TDI-HSA ^b	12/19 (63.2%)	20/72 (27.8%)	0.004 ^d
sIgG to TDI-HSA ^b	8/19 (42.1%)	19/71 (26.8%)	0.644 ^d

TDI-OA toluene diisocyanate-induced occupational asthma, tTG tissue transglutaminase, FEV₁ forced expiratory volume in 1 s, PC₂₀ concentration of methacholine required to produce a 20% decrease in FEV₁, sIgE serum-specific IgE antibodies, TDI-HSA toluene diisocyanate-human serum albumin, sIgG serum-specific IgG antibodies

^a Indicate variables tested by *t* test

^b Indicate variables tested by χ^2 test

^c Adjusted for age and sex using multiple linear regression

^d Adjusted for age and sex using multiple logistic regression

lar ROS production in response to TDI-HSA using the cell-permeable ROS-sensitive fluorophore, 2', 7'-dichlorodihydrofluorescein diacetate. Intracellular ROS production increased significantly by TDI-HSA in a dose-dependent manner. Also, intracellular ROS production increased significantly within 15 min and peaked at 60 min (Fig. 3a, b). As shown in Fig. 4, when A549 cells pretreated with *N*-acetylcysteine, a ROS scavenger, were incubated with TDI-HSA, TDI-HSA-induced increases in tTG activity were attenuated to basal levels, suggesting that TDI-HSA-induced increases in tTG activity are mediated by TDI-HSA-induced ROS production.

The Cross-linking Between tTG and CK19

To evaluate the association between CK19 and tTG activity, we first analyzed the change in intracellular tTG localization by Western blot analysis. As shown in Fig. 5a, when A549 cells were treated with TDI-HSA, tTG protein was increased, especially that in insoluble fraction, suggesting that activated tTG may interact with detergent-insoluble proteins or detergent-insoluble aggregates. CK19 is a well-known detergent-insoluble protein, and we detected a large amount of CK19 in the detergent-insoluble fraction; only trace amounts were present in the detergent-soluble fraction in A549 cells. To determine whether activated tTG induces

CK19 cross-linking, we performed an in vitro cross-linking assay. Figure 5b shows that tTG induced the formation of high molecular weight CK19 aggregates in the presence of calcium chloride, a known activator of tTG, in a dose-dependent manner. In contrast, chelation by EDTA blocked aggregate formation, suggesting that CK19 is a target for tTG in vitro.

Discussion

Previous studies indicated that IgE-mediated response to TDI exposure may play a key role in the pathogenesis of

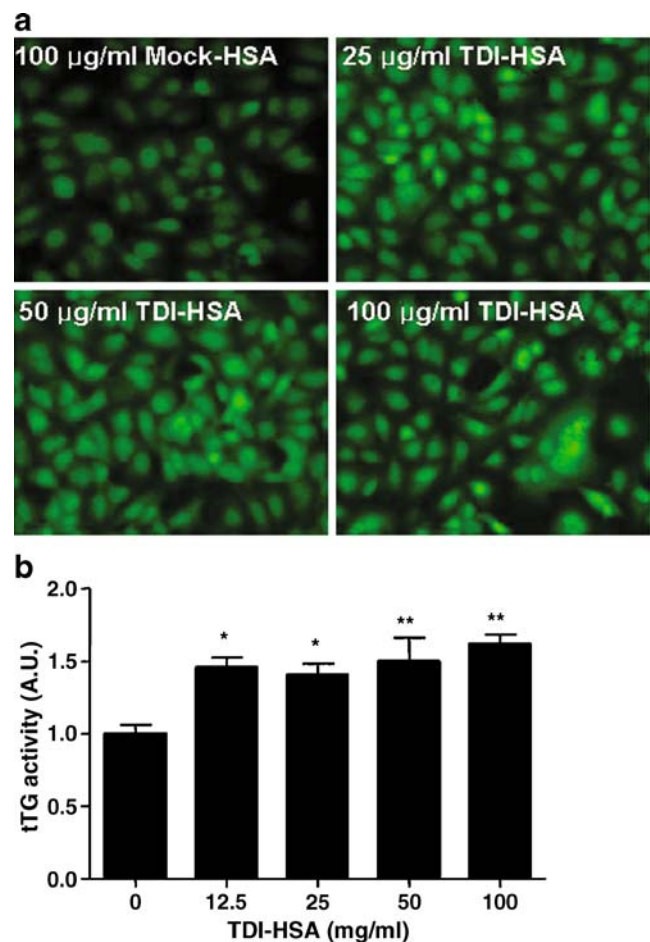


Fig. 2 Activation of tissue transglutaminase (tTG) with exposure to toluene diisocyanate-human serum albumin (TDI-HSA) conjugate in A549 cells. **a** Cells were incubated with 1 mM 5-(biotinamido) pentyamine for 1 h and then stimulated with the indicated concentrations of TDI-HSA for 3 h. Cells were stained with FITC-conjugated streptavidin and observed using fluorescence microscope. The fluorescent images show localized in situ tTG activity. Scale bar, 20 µm. **b** The fluorescent intensities of the cells in **a** were counted using an image browser, and the data were presented as A.U. as described in “Materials and Methods”. Data are the representative of three independent experiments and were analyzed using the Mann-Whitney *U* test. Values represent the mean ± standard error. **P*<0.05 vs. control; ***P*<0.01 vs. control

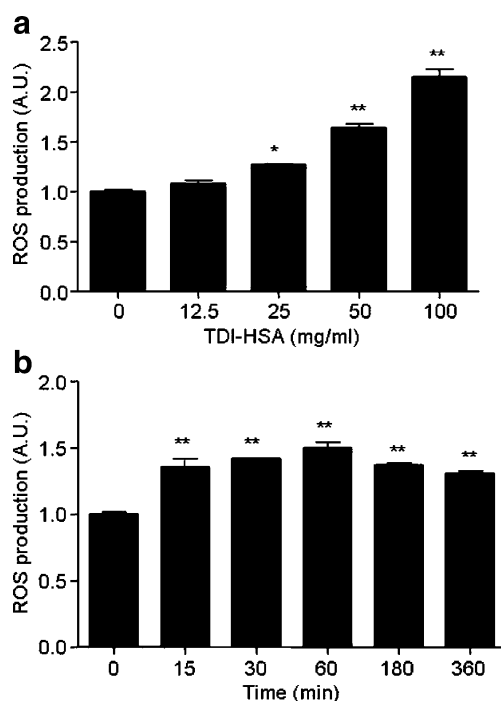


Fig. 3 Measurement of intracellular reactive oxygen species (ROS) production induced by toluene diisocyanate–human serum albumin (TDI–HSA) in A549 cells. **a** Cells were incubated with 5 μ M 2',7'-dichlorodihydrofluorescein diacetate for 30 min at 37°C and with the indicated doses of TDI–HSA for 60 min. **b** Cells were incubated with 100 μ g/mL TDI–HSA for the indicated times and then analyzed by flow cytometry as described in “Materials and Methods”. Data were analyzed using the Mann–Whitney *U* test. Values are mean $\pm$ SE. **P* < 0.05 vs. control, ***P* < 0.01 vs. control

TDI–OA; however, in previous studies, the prevalence of serum-specific IgE to TDI–HSA varied between 0% and 40% [5, 17–19]. In this study, a vapor-type TDI–HSA conjugate [6] that shows a higher immunogenic potential than observed in previous studies [5, 14] was used in in vitro experiment. The relationship between specific IgE to this conjugate and IgG to tTG level was observed.

Many stimuli, including isocyanate and proinflammatory cytokines, induce cytokine and chemokine production and cellular recruitment in bronchial epithelial cells [3, 20, 21]. TDI-generated ROS may induce barrier dysfunction in epithelial cells and reduce antioxidant levels [22–25]. A recent study demonstrated that ROS may play a role in the pathogenesis of airway inflammation and asthma [26]. Cells obtained from peripheral blood and the lungs of asthmatic patients generate increased amounts of ROS, which are strongly correlated with disease severity [27–30]. In mice, ROS generation in bronchoalveolar lavage fluid increased after TDI inhalation compared to saline inhalation [31]. In TDI–OA patients, activated neutrophils appear to play a role in airway inflammation [8, 32]. Our in vitro experiments demonstrated that TDI exposure induces ROS generation in airway epithelial cells which were suppressed

by a ROS scavenger suggesting that TDI-induced airway inflammation can be partly explained by the properties of ROS production.

Evidence suggests that tTG plays an active role in various biological functions, including the induction of apoptosis and cell adhesion [33]. In addition, the presence of autoantibodies against tTG and its protein substrates in autoimmune diseases suggests that tTG may cross-link potential autoantigens to itself and other protein substrates,

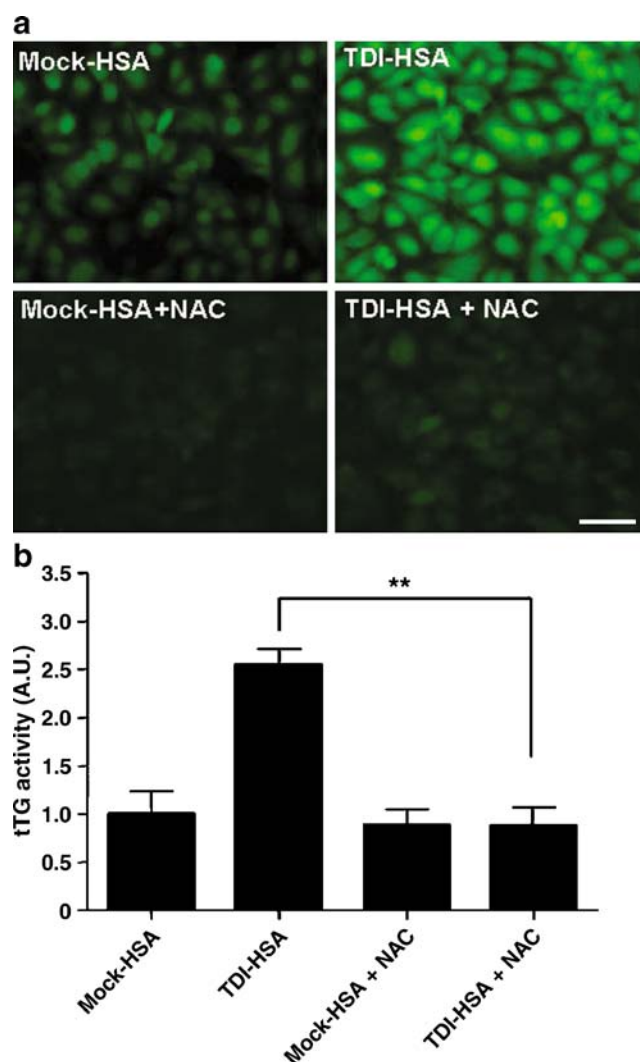


Fig. 4 Activation of tissue transglutaminase (tTG) by toluene diisocyanate–human serum albumin (TDI–HSA) via reactive oxygen species (ROS) production in A549 cells. **a** Cells were incubated with 1 mM 5-(biotinamido)pentylamine for 1 h and then stimulated with 100 μ g/mL TDI–HSA or equivalent dose of mock HSA as control in the presence or absence of 20 mM *N*-acetylcysteine (NAC) for 3 h. Cells were stained with FITC-conjugated streptavidin and observed using a fluorescence microscope. Scale bar, 20 μ m. **b** The fluorescent intensities of the cells in **a** were counted using an image browser, and the data were presented as arbitrary unit (A.U.) as described in “Materials and Methods”. Data are representative of three independent experiments and were analyzed by Mann–Whitney *U* test. Values represent the mean $\pm$ standard error. ***P* < 0.01 vs. control

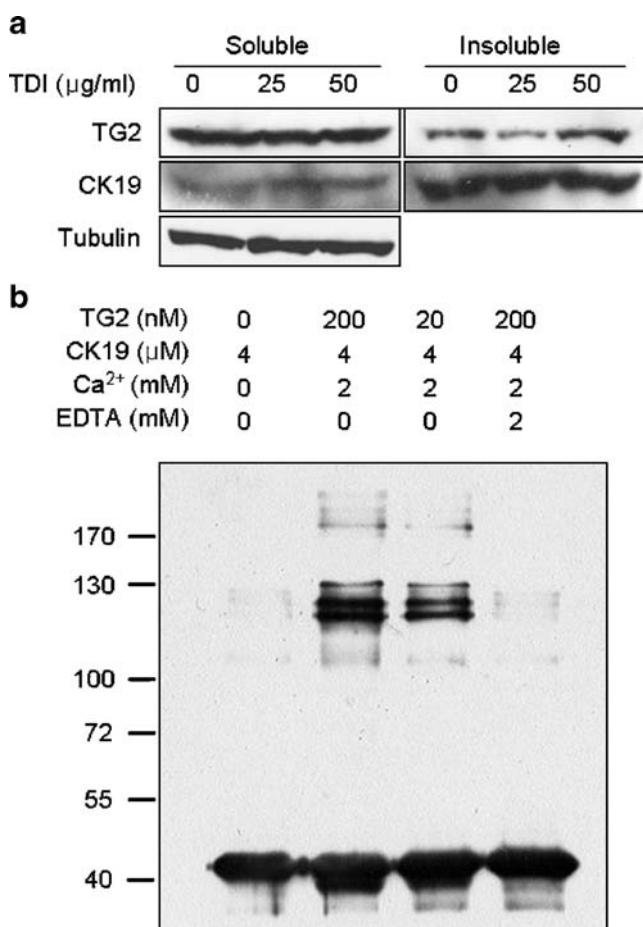


Fig. 5 Immunoblot analysis of bronchial epithelial cells using tissue transglutaminase and cytokeratin 19 (CK19). **a** A549 cells were incubated with the indicated concentrations of toluene diisocyanate–human serum albumin (TDI–HSA) for 24 h, lysed, separated into soluble and insoluble fractions as described in “Materials and Methods”, and subjected to Western blot analysis. **b** Recombinant CK19 was incubated with guinea pig tTG for 1 h at 37°C under the conditions indicated, and Western blot analysis was performed using anti-CK19 antibody. Data are representative of three independent experiments

thus triggering a humoral immune response in autoimmune diseases [34, 35]. In the present study, the prevalence of serum IgG to tTG in TDI-OA patients was significantly higher than that in the AEC, AA, or NC. Because none of AA patients had high IgG to tTG levels, these results may be specific to TDI-OA, as opposed to asthma in general or allergen-induced airway inflammation. Airway hyperresponsiveness to methacholine was significantly lower in TDI-OA subjects with high IgG to tTG levels than those without IgG to tTG. In addition, the *in vitro* experiments demonstrated that TDI exposure generates ROS, which in turn activates tTG. Thus, we speculate that TDI exposure activates tTG and promotes the production of IgG to tTG which may be involved in airway inflammation. Although the prevalence of serum IgG to tTG was not sufficiently high (20.2%) to be used as a diagnostic marker for TDI-

OA, it may be helpful to diagnose TDI-OA among the TDI-exposed workers.

In our previous report [36], microarray analysis has demonstrated the increased expression of CK19 messenger RNA from culture of bronchial epithelial cells exposed to TDI. The prevalence of IgG to CK19 was significantly higher in TDI-OA patients than in AEC or NC. Furthermore, we observed a significant association between the presence of IgG to CK19 and IgG to tTG ($P=0.02$). Cytokeratins are abundantly present in the intracellular space and gain access to the immune system after epithelial damage or cell death [37]. tTG is also present in bronchial epithelial cells and transforms proteins by deamidation and/or transamidation as suggested in several autoimmune diseases [38–40]. Based on these findings, we hypothesized that tTG may cross-link CK19, which may influence the development of antibodies against tTG and/or CK19. To determine whether tTG interacts with CK19, we performed an immunoblot analysis and confirmed the presence of cross-linking, suggesting that CK19 is a substrate for tTG. This result is supported by previous data that the glutamine (Gln) residue of target proteins may influence tTG activity. Sequences preferred by tTG are Gln-X-Pro or Gln-X-Pro(Ile, Leu, Val, The, Tyr, Trp, Thr, or Ser; X denotes any amino acid) [41]. There exist the tTG-preferred sequences in that of CK19 (119Q, OGP). This result is also consistent with a previous study showing that hepatocellular cytokeratins serve as substrates for tTG [42]. Based on these findings, we suggest that TDI exposure induces ROS production and activates tTG which may play a role to cross-link with various substrate proteins such as CK19. Subsequently, tTG, CK19, and substrate proteins may trigger humoral immune responses which lead to airway inflammation in patients with TDI-OA.

We noted significant associations and correlations between the prevalence of serum IgG to tTG and that of specific IgE to TDI–HSA conjugate in TDI-exposed subjects. Moreover, patients with detected IgG to tTG levels had a significantly higher prevalence of serum-specific IgE than those without detectable levels (63.2% vs. 27.4%; $P=0.004$). Given that tTG has deamidase activity and may generate new epitopes, tTG activity may enhance the production of specific IgE to TDI–HSA in exposed workers, as suggested by a previous study on food allergies [43], which may be associated with airway inflammation and development of TDI-OA.

Several limitations were raised in this study. The preparation method of TDI–HSA conjugate and ELISA for detecting serum-specific antibodies to TDI–HSA conjugate is not well standardized between the different laboratories. Moreover, all the *in vitro* studies were done using bronchial epithelial cell lines. Therefore, further

investigations were needed to understand the exact mechanism by which tTG can activate CK and augment specific IgE response using primary bronchial epithelial cells from the patient.

In conclusion, TDI exposure could activate tTG via a ROS-mediated mechanism in the airway epithelium which could enhance IgE production to tTG and airway inflammation in TDI-OA patients.

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