Epithelial folliculin is involved in airway inflammation in workers exposed to toluene diisocyanate

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Toluene diisocyanate (TDI) exposure can directly activate and damage airway epithelium. Folliculin (FLCN) is a protein expressed by human airway epithelial cells (HAECs) to maintain airway epithelial integrity and survival. This study investigated the involvement of FLCN in the pathogenesis of TDI-induced occupational asthma (OA). Enzyme-linked immunosorbent assay was used to measure serum levels of FLCN in TDI-exposed subjects (93 TDI-OA patients and 119 asymptomatic exposed controls (AEC)), 200 non-occupational asthma (NOA) patients and 71 unexposed healthy normal controls (NCs). Significantly more subjects in the TDI-OA and AEC groups had high serum levels of FLCN compared to those in the NOA group ($P=0.002$ and $P=0.001$, respectively), all of which were higher than the NC group (all $P<0.001$). The serum level of FLCN was positively correlated with TDI exposure duration ($r=0.251$, $P=0.027$), but was negatively correlated with asthma duration of TDI-OA patients ($r=-0.329$, $P=0.029$). TDI-exposed subjects with high FLCN levels had higher serum levels of total IgE than those with lower levels. The effects of TDI exposure on FLCN production was investigated by treating HAECs (A549 cells) with TDI-human serum albumin conjugate, which showed increased expression and release of FLCN and interleukin-8 from HAECs. Co-culture with peripheral blood neutrophils also induced FLCN expression and release from HAECs. In conclusion, TDI exposure and TDI-induced neutrophil recruitment into the airways can activate and stimulate HAECs to produce FLCN, which could be involved in airway inflammation in workers exposed to TDI.

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INTRODUCTION

Toluene diisocyanate (TDI) is a low molecular weight compound used in the production of polyurethane foams, automobile paint, varnishes and related products. Long-term exposure to TDI can lead to the development of TDI-induced occupational asthma (OA), a common OA in industrialized countries worldwide.\textsuperscript{1} TDI-OA has a poor prognosis and its pathogenesis is not completely understood, but could be more complicated than that of non-occupational asthma (NOA). Exposure to TDI directly damages airway epithelium, leading to increased airway epithelial permeability. Moreover, TDI exposure activates airway epithelial cells, which produce cytokines and recruit inflammatory cells such as neutrophils into the airways, which then enhances the airway inflammation and leads to a severe asthma phenotype.\textsuperscript{2,3}

Folliculin (FLCN) is expressed in the skin, kidneys, and upper and lower airway epithelial cells.\textsuperscript{4} Mutations in the FLCN gene have been implicated in Birt–Hogg–Dube syndrome, which is characterized by lung cysts and spontaneous pneumothorax.\textsuperscript{5,6} Recent studies have reported the role of FLCN in maintaining the integrity and function of airway epithelial cells and lung fibroblasts. Knockout of FLCN induced apoptosis, downregulated the expression of E-cadherin in mouse lung alveolar cells and damaged several signaling pathways in human airway epithelial cells (HAECs).\textsuperscript{7,8} These findings suggest that FLCN may involve in chronic airway diseases that involve airway damage. However, to date, the involvement of FLCN in OA and NOA is unknown.

In this study, we measured the serum levels of FLCN in patients with TDI-OA, asymptomatic TDI-exposed controls (AECs), NOA patients and unexposed normal healthy controls (NCs) to evaluate the involvement of FLCN in these diseases. We also investigated the effects of TDI and neutrophil inflammation on HAEC activation and their production of FLCN.
MATERIALS AND METHODS

Subject recruitment

We recruited 212 TDI-exposed workers (including 93 TDI-OA patients and 119 AECs), 200 NOA patients and 71 NCs from Ajou University Medical Center (Suwon, South Korea). TDI-OA was diagnosed by a positive response to a TDI bronchoprovocation test, as previously described. A diagnosis of Birt–Hogg–Dube syndrome was excluded based on disease history and chest X-ray. One week before serum collection, the TDI-OA and NOA patients stopped using leukotriene modifiers and anti-inflammatory agents, including inhaled or oral corticosteroid. Serum samples were collected at the time of diagnosis and stored at −70 °C until use. Atopy status was defined as one or more positive reactions on skin prick tests with 55 common aeroallergens (Bencard, Bradford, UK). All of the study subjects provided written informed consent. The study was approved by the Institutional Review Board of Ajou University Medical Center.

Detection of serum-specific IgG and sIgE antibodies to TDI-human serum albumin conjugate by enzyme-linked immunosorbent assay

Vapor-type TDI-human serum albumin (TDI-HSA) and mock-HSA conjugates were kindly provided by Dr Adam Wisnewski (Yale University, New Haven, CT, USA). Serum-specific IgG (sIgG) and serum-specific (sIgE) antibodies to TDI-HSA were detected using a homemade enzyme-linked immunosorbent assay (ELISA), as previously described.

ELISA to measure serum levels of FLCN and interleukin-8

Commercial ELISA kits were used to measure the serum levels of FLCN (CUSABIO Biotech, Wuhan, Hubei Province, China) and interleukin-8 (IL-8) (Endogen, Woburn, MA, USA), as well as the levels in cell culture supernatants, following the manufacturer’s protocols.

Isolation of peripheral blood neutrophils

Blood samples were collected from healthy donors into BD Vacutainer tubes containing acid citrate dextrose solution (BD Biosciences, Franklin Lakes, NJ, USA), stored at room temperature (RT), and were processed within 2 h of collection. Peripheral blood neutrophils (PBNs) were isolated by gradient centrifugation on Lymphoprep solution (Axis-Shield, Oslo, Norway), followed by sedimentation in Hank’s balanced salt solution buffer containing 2% dextran (Polysciences, Warrington, PA, USA), as previously described. Cell viability (>98%) was assessed by trypan blue staining. Cell purity (>95%) was assessed by hematoxylin and eosin (H&E) staining and flow cytometry using CD68 and CD11b expression.

HAEC culture and treatment

A human lung carcinoma cell line (A549) was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin G sodium and 100 μg ml⁻¹ streptomycin sulfate (all from Gibco, Grand Island, NY, USA). Cells were maintained at 37 °C with 5% CO₂ in humidified air. Cells (2 × 10⁴) were seeded onto each well of a 12-well plate (TPP, Trasadingen, Switzerland) and treated with 2–200 μg ml⁻¹ TDI-HSA in serum-free RPMI-1640 medium. Mock-HSA conjugate was used as a control (data not shown). In co-culture experiments, different numbers of PBNs were added onto A549 cells in serum-free medium. After a 24 h incubation, the supernatant was collected, and the cells were lysed in a radioimmunoprecipitation assay buffer and stored at −70 °C for further experiments.

Western blotting to detect FLCN

The proteins (30 μg) from A549 cell lysates were loaded onto 10% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA, USA). After blocking in 5% skim milk (Sigma, St Louis, MO, USA) in PBS containing 0.05% Tween 20 (PBS-T) for 1 h at RT, the membranes were incubated with rabbit anti-human FLCN antibody (Cell Signaling, Minneapolis, MN, USA) overnight at 4 °C. Then the membranes were washed three times with PBS-T for 10 min each, and incubated with the appropriate secondary antibody for 1 h at RT. The membranes were blotted with anti-beta actin as a loading control. Signals were detected using ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). The intensity of bands was analyzed using a gel doc system (Bio-Rad).

Statistical analysis

The serum levels of FLCN in the study subjects were log-transformed before statistical analysis to establish a normal distribution. Data for continuous variables were compared using the Student’s t-test or Mann–Whitney U-test; Pearson’s χ² or Fisher’s exact tests were used for categorical variables. Statistical correlations were analyzed using Pearson’s coefficient or Spearman’s rank coefficient. All of the statistical analyses were performed with SPSS ver. 22.0 (SPSS, Chicago, IL, USA). P values <0.05 were considered statistically significant. GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) was used for graphs, with values presented as the mean ± standard deviation (s.d.) of at least three independent experiments.

RESULTS

Clinical characteristic of the study subjects

Females predominated in the NOA (60.0%) and NC (60.6%) groups, whereas males predominated in the TDI-OA (69.9%) and AEC (68.1%) groups (Table 1). The duration of TDI exposure of TDI-OA patients (6.19 ± 4.3 years) was significantly shorter than that of AEC group (12.05 ± 8.2 years, P = 0.003). The prevalence of sIgG and sIgE antibodies against TDI-HSA in the TDI-OA group was significantly higher than that in the AEC group (23.8% vs 5.9% and 23.5% vs 1.7%, respectively; both P < 0.001). No differences in serum levels of total IgE or asthma duration were observed between the TDI-OA and NOA groups.

Elevated serum levels of FLCN in TDI-exposed subjects and NOA patients

We log-transformed the serum level of FLCN to correct its skewed distribution. Serum levels of FLCN (Figure 1a) of the TDI-OA (1.57 ± 0.31), AEC (1.64 ± 0.31) and NOA (1.50 ± 0.24) groups were all significantly higher than those of the NC group (1.31 ± 0.21, all P < 0.001). The serum levels of FLCN in both the TDI-OA and AEC groups were significantly higher than those in the NOA group (P = 0.038 and P < 0.001, respectively). We classified the study subjects into two groups with high (FLCN-high) and low (FLCN-low) serum levels of FLCN using a cutoff value (1.75) calculated as the mean plus two s.d.’s of the serum level of FLCN.
The prevalence of FLCN-high subjects in the TDI-OA (33.3%) and AEC (38.7%) groups did not significantly differ (P = 0.473), whereas both were significantly higher than the NOA group (16.5%, P = 0.002 vs TDI-OA and P < 0.001 vs AEC). The prevalence of FLCN-high subjects in the NC group (4.2%) was significantly lower than that in the other three groups (P < 0.001 for all). Consequently, TDI-exposed subjects (including TDI-OA and AEC subjects) had significantly higher serum levels of FLCN (1.61 ± 0.31) and significantly higher prevalence of FLCN-high subjects (36.3%) than the NOA and NC groups (all P < 0.001, Figure 1b).

**Table 1 Clinical characteristic of the study subjects**

|                      | TDI-OA (n = 93) | AEC (n = 119) | NOA (n = 200) | NC (n = 71) | P-value |
|----------------------|-----------------|---------------|---------------|-------------|---------|
| Agea                 | 42.8 ± 10.5     | 40.57 ± 8.7   | 44.66 ± 15.8  | 39.75 ± 14.0| 0.122   |
| Sex (female)b        | 28 (30.1)       | 38 (31.9)     | 60 (60.0)     | 43 (60.6)   | 0.776   |
| Atopyb               | 38/77 (49.35)   | NA            | 89/186 (47.8) | NA          | NA      |
| FEV1 (% predicted)a  | 84.7 ± 21.78    | 90.17 ± 20.24 | 89.29 ± 20.78 | NA          | 0.476   |
| MchPC20 (mg ml−1)b   | 7.04 ± 13.39    | 7.94 ± 9.35   | 12.42 ± 12.04 | NA          | 0.266   |
| Serum total IgE (IU l−1)c | 327.2 ± 497.9    | 231.05 ± 618.9 | 409.75 ± 719.16 | NA          | 0.097   |
| TDI exposure duration (years)d | 6.19 ± 4.3     | 12.05 ± 8.2   | NA            | NA          | 0.003   |
| Asthma duration (years)d | 6.68 ± 4.11     | NA            | 7.2 ± 6.58    | NA          | 0.001   |
| slgG to TDI-HSAc      | 19/80 (23.8)    | 7/119 (5.9)   | NA            | NA          | 0.001   |
| slgE to TDI-HSAc      | 19/81 (23.5)    | 2/119 (1.7)   | NA            | NA          | 0.001   |

**Abbreviations:** AEC, asymptomatic TDI-exposed control; FEV1, forced expiratory volume in 1 s; MchPC20, concentration of methacholine required to produce a 20% decrease in FEV1; NA, not available; NC, normal healthy control; NOA, non-occupational adult asthma; slgG and slgE, serum-specific IgG and IgE antibody; TDI-HSA, toluene diisocyanate-human serum albumin conjugate; TDI-OA, TDI-induced occupational asthma.

aData are shown as the mean ± s.d., P-value obtained with the Mann–Whitney U-test.

bData are shown as the prevalence (%), P-value obtained with the χ2 test.

Bold numbers indicate statistically significant values.

**Figure 1** Comparison of serum FLCN levels among the four study groups (a) and among the TDI-exposed subjects (including TDI-OA and AEC groups), NOA and NC groups (b). Dot plots show the log-transformed serum FLCN level. Horizontal dashed lines indicate the cutoff value, and horizontal solid lines in the middle of the dot plots indicate the mean value of each group. The P-value was obtained using Fisher’s exact test to compare the prevalence of subjects with high serum FLCN levels (%) among the indicated groups. AEC, asymptomatic TDI-exposed controls; FLCN, folliculin; NC, normal healthy controls; NOA, non-occupational adult asthma; TDI-OA, TDI-induced occupational asthma.

**Associations of serum levels of FLCN with clinical parameters**

We observed that the serum levels of FLCN were positively correlated with TDI-exposure duration (r = 0.251, P = 0.027, Figure 2a), but were negatively correlated with asthma duration of the TDI-OA patients (r = −0.329, P = 0.029, Figure 2b). Nevertheless, no significant correlation between serum levels of FLCN and asthma duration of NOA group was noted (r = −0.182, P = 0.295). The TDI-exposed FLCN-high subjects were also significantly older (P = 0.029) and had higher serum levels of total IgE (P = 0.021) than the FLCN-low subgroup (Table 2). No associations of serum levels of FLCN with the
Table 2 Comparison of the clinical and immunological features according to the level of serum FLCN in TDI-exposed subjects

| Serum FLCN level | High (n = 77) | Low (n = 135) | P-value |
|------------------|---------------|---------------|---------|
| Age (years)a     | 43.55 ± 9.75/75 | 40.38 ± 9.24/133 | 0.029   |
| Atopyb           | 16/24 (66.7)   | 24/54 (44.4)   | 0.088   |
| Sex (female)b    | 20/77 (26.0)   | 46/135 (34.1)  | 0.280   |
| Asthma duration (years)a | 5.92 ± 4.03/12 | 6.97 ± 4.16/32 | 0.524   |
| FEV1 (% predicted)a | 81.06 ± 15.85/22 | 87.79 ± 23.49/47 | 0.168   |
| MchPC20 (mg ml−1)a | 6.27 ± 11.76/23 | 7.53 ± 13.36/56 | 0.791   |
| Serum total IgE  | 410.5 ± 723.9/40 | 241.3 ± 401.2/78 | 0.021   |
| Serum sIgG to TDI-HSA | 6/71 (8.5)   | 20/128 (15.6)  | 0.190   |
| Serum sIgE to TDI-HSA | 8/72 (11.1)  | 13/128 (10.2)  | 0.815   |
| Serum IL-8 (pg ml−1)a | 48.06 ± 72.89/69 | 58.9 ± 139.1/127 | 0.777   |

Abbreviations: FEV1, forced expiratory volume in 1 s; IL-8, interleukin 8; MchPC20, concentration of methacholine required to produce a 20% decrease in FEV1; sIgG and sIgE, serum-specific IgG and IgE antibody; TDI-HSA, toluene diisocyanate-human serum albumin conjugate

Data are shown as the mean ± s.d., P-value obtained with the Mann-Whitney U test.

Discussions

Neutrophils induce FLCN production from A549 cells
Neutrophilic airway inflammation contributes to TDI-OA development.2 Because TDI-HSA induced A549 cells to produce IL-8, a strong neutrophil chemoattractant, we evaluated the role of neutrophils in FLCN production by HAECs. A549 cells were co-cultured with PBNs at different cell number ratios. We observed that PBNs significantly increased both the intracellular expression (Figure 4a) and extracellular release (Figure 4b) of FLCN from A549 cells in a dose-dependent manner.

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Effect of TDI exposure on FLCN production from HAECs
When A549 cells were treated with 2–200 μg ml−1 TDI-HSA conjugate, TDI-HSA dose-dependently increased both the intracellular expression (Figure 3a) and extracellular release (Figure 3b) of FLCN compared to mock-HSA treatment. TDI-HSA also induced IL-8 production from A549 cells in a dose-dependent manner (Figure 3c), which was strongly correlated with the FLCN level released from those cells (r = 0.779, P = 0.003, Figure 3d). TDI-HSA did not affect A549 cell viability at the tested concentrations (Figure 3e).

Prevalence of serum sIgE or sIgG to TDI-HSA or serum levels of IL-8 were observed.

DISCUSSION

Toluene diisocyanate exposure directly damaged HAECs and activated their inflammatory response, leading to airway epithelium impairment and the infiltration of inflammatory cells into the airways.2 FLCN is a protein expressed by airway epithelial cells that regulates the survival and adherence of alveolar epithelial cells.7 Recently, FLCN has been detected in the bronchial alveolar lavage fluid from asthmatic patients, suggesting a role in asthma.12 Nevertheless, the function of FLCN in asthma and its involvement in TDI-OA have not been studied. This is the first study to describe increased serum levels of FLCN in TDI-exposed workers, including TDI-OA and AEC groups, as well as NOA patients compared to normal healthy subjects. We also found that TDI exposure induced the concomitant release of FLCN and IL-8 from A549 cells, which was also induced by co-culture of A549 cells with PBNs. These findings suggest the involvement of FLCN in airway inflammation in TDI-exposed subjects.

A previous study found increased FLCN secretion from compressed human bronchial epithelial cells,12 suggesting that FLCN is released from HAECs in asthmatic patients. Consistently, we found higher serum FLCN levels in both TDI-OA and NOA patients compared to healthy subjects, implicating FLCN in the pathogenesis of asthma. We also observed higher production of serum FLCN in TDI-OA and AEC subjects compared to the NOA and NC groups, as well as a positive correlation of serum FLCN levels with TDI-exposure duration, but not with the asthma duration of NOA patients. These findings suggest that TDI is a stronger inducer of FLCN
The role of FLCN in TDI-OA and asthma has not been investigated, although several studies have suggested that FLCN has a protective function maintaining the integrity and survival of HAECs. Moreover, serum levels of FLCN were negatively correlated with disease duration of the TDI-OA patients, which suggests that long-term airway epithelial cell damage induced by TDI could lead to the reduced production of FLCN. Overall, we speculate that increased expression or secretion of FLCN upon TDI exposure is not only a consequent response but also a protective mechanism against TDI-induced airway damage. The function of FLCN in TDI-OA and NOA remains to be investigated.

The consequences of the aging process have been implicated in innate immune responses, including HAEC dysfunction and increased neutrophils infiltrating into airways of asthmatics. We observed that TDI-exposed subjects who had high serum levels of FLCN were older than FLCN-low subjects, suggesting that the aging process affects FLCN production from HAECs. Moreover, airway epithelium is a barrier to the infiltration of allergens into the subepithelial layers where they interact with antigen-presenting cells, which could regulate allergen-specific antibody production. Therefore, the association of serum levels of FLCN and total serum levels of IgE observed in this study may be an epiphenomenon that resulted from airway damage and increased airway permeability induced by TDI exposure.

To validate our hypothesis that TDI exposure induces FLCN production from HAECs, we treated A549 cells with TDI-HSA conjugate, which dose-dependently increased both the intracellular expression and extracellular release of FLCN. Interestingly, the extracellular FLCN levels strongly correlated with IL-8 production from A549 cells, suggesting that FLCN is an activation marker of HAECs. Furthermore, TDI-HSA conjugate stimulated A549 cells to produce IL-8, which could be considered an activation maker of HAECs. Interestingly, the extracellular FLCN levels strongly correlate with IL-8 production from A549 cells, suggesting that FLCN is an activation marker of HAECs.
correlated with the IL-8 levels. These findings suggest that TDI exposure activates HAECs to produce FLCN, explaining the elevated serum FLCN levels in TDI-exposed subjects. However, the increased FLCN level in NOA patients could be a consequence of bronchial contraction, whereas that in TDI-exposed subjects is induced by direct stimulatory effects of TDI. The mechanisms underlying the TDI-induced FLCN expression need further investigation.

Previously, we reported higher numbers of neutrophils infiltrating the airways of TDI-OA patients compared to AEC and NOA subjects, suggesting the contribution of neutrophils to the development of airway inflammation in TDI-OA. Moreover, we consistently found that TDI exposure stimulates HAECs to produce IL-8, a well-known neutrophil chemoattractant. Consequently, we elucidated the role of neutrophils in FLCN production from HAECs in this study. Co-culture with PBNs significantly increased FLCN expression and release from A549 cells, suggesting that TDI-induced neutrophil infiltration of the airway enhances the production of FLCN by HAECs. However, we did not find any correlation between serum levels of FLCN and sputum neutrophil count or serum levels of IL-8 in TDI-exposed subjects. Perhaps FLCN production in TDI-exposed subjects is also affected by various factors that participate in the airway inflammation process.

In addition, we observed the increased serum FLCN level from TDI-exposed subjects regardless the development of TDI-OA. It is known that the development of TDI-OA depends on various mechanisms, including immunologic, non-immunologic and genetic molecular mechanisms. We speculate that FLCN is known that the development of TDI-OA depends on various immunologic, non-immunologic and genetic molecular mechanisms. We speculate that FLCN is a key regulator of airway allergen sensitization and remodeling in severe asthma.

In conclusion, TDI exposure and TDI-induced neutrophil recruitment into the airways directly activates HAECs and stimulates them to produce FLCN, could be involved in airway inflammation in workers exposed to TDI.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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