

## ORIGINAL ARTICLE

# Toluene diisocyanate enhances human bronchial epithelial cells' permeability partly through the vascular endothelial growth factor pathway

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## Clinical & Experimental Allergy

### Summary

**Background** Toluene diisocyanate (TDI) is a recognized chemical asthmogen; yet, the mechanisms of its toxicity have not been elucidated.

**Objective** To investigate the influence of TDI on the permeability of human bronchial epithelial cell (HBE; HBE135-E6E7) monolayers *in vitro*, and the expression of vascular endothelial growth factor (VEGF) in these cells.

**Methods** TDI-human serum albumin (HSA) conjugates were prepared by a modification of Son's method. Fluorescein isothiocyanate-labelled dextran and transmission electron microscopy were used to evaluate the effects of TDI-HSA on HBE135-E6E7 permeability. RT-PCR and ELISA were used to evaluate VEGF gene expression and protein release from HBE135-E6E7 cells stimulated by TDI-HSA. A VEGF-neutralizing antibody was used in monolayer permeability experiments to determine the role of the VEGF pathway in this process.

**Results** TDI-HSA significantly increased the permeability coefficients of HBE135-E6E7 monolayers ( $P < 0.01$ ). TDI-HSA treatment significantly increased the expression of VEGF165 and VEGF189 genes ( $P < 0.01$ ). ELISA showed that TDI significantly induces VEGF release from HBE135-E6E7 cells. Cells treated with TDI-HSA and VEGF-neutralizing antibody had significantly lower permeability coefficients than cells treated with TDI-HSA only ( $P < 0.01$ ), but still significantly higher than control cells ( $P < 0.01$ ). Cells treated with TDI-HSA had fewer tight junctions (TJs) than control and HSA-treated cells, and addition of the anti-VEGF antibody did not restore the original number of TJs.

**Conclusion** TDI increases the permeability of HBE cell monolayers, partly through a VEGF-mediated pathway. This suggests the importance of VEGF in TDI-induced pulmonary diseases, but shows that other pathways may be involved in the pathogenic process.

**Keywords** asthma, cell permeability, human bronchial epithelial (HBE) cells, toluene-2,4-diisocyanate (TDI), vascular endothelial growth factor (VEGF)

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### Introduction

The airway epithelium functions as a barrier that protects the underlying tissue against allergens, irritants, viruses, inhaled toxic substances [such as toluene diisocyanate (TDI)], and microbial pathogens. Airway epithelium cells (AECs) are central to the pathogenesis of asthma and other chronic airway diseases, in which the barrier function of the epithelium is impaired due to the formation of

defective tight junctions (TJs) [1, 2]. The epithelial cell layer forms a highly regulated and impermeable barrier through the formation of TJs, and acts as a molecular sieve that excludes inhaled antigens, pathogens, and extrinsic protein based on their molecular weight. Several lines of experimental evidence indicate that all proteins are absorbed across mucosal epithelia by transcellular transport and/or through interstitial spaces in the epithelial cells, but not at equal levels [2, 3].

TDI, a highly reactive industrial chemical with widespread use in the manufacture of polyurethane and plastics, is a recognized chemical asthmogen; like other low-molecular-weight chemicals, it is not antigenic *per se*

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(it is a hapten), but it is spontaneously reactive with a variety of proteins, such as albumin, keratin, haemoglobin, and ciliated cell tubulin, and forms immune conjugates [4–8]. Thus, molecular characterization of the adducted biomolecules should help understand the pathogenesis of TDI-induced respiratory diseases.

Ogawa et al. [9] found that TDI–human serum albumin (HSA) conjugate induces the production of inflammatory cytokines and chemokines by human bronchial epithelial cells (HBEs) via the epidermal growth factor receptor and p38 mitogen-activated protein kinase pathways. One study [8] demonstrated that treatment of primary HBE cells with low concentrations of TDI (20–100 p.p.b.) for 30 min did not significantly influence the barrier function of the epithelium. Nevertheless, the precise contribution of TDI to AEC permeability and its regulation of ring joints is still unknown.

Vascular endothelial growth factor (VEGF) is a mediator of airway inflammation and remodelling in asthma. Overexpression of VEGF under the control of a lung-specific promoter increases vascular permeability (causing oedema) in asthmatics, and results in Th2 cell sensitization to otherwise harmless antigens [10]. This suggested that VEGF regulation may be therapeutic in asthma and other Th2-related disorders. It was also suggested that VEGF may be one of the major determinants of TDI-induced asthma in murine models of TDI-induced asthma, and that inhibition of VEGF may be a good therapeutic strategy [11, 12]. Furthermore, numerous reports have shown increases in lung vasculature in asthmatics, and a strong correlation between the degree of vascular abnormalities and asthma severity [13, 14]. Increased expression of VEGF in the induced sputum of TDI-induced asthma patients has been reported [15], and AECs are the most important source of VEGF [16].

The purpose of the present study was to assess the influence of TDI–HSA on the permeability of epithelial cells monolayers and the expression of VEGF in HBE cells *in vitro* to determine whether VEGF mediates the regulation of epithelial cell monolayer permeability.

Our findings show that TDI–HSA significantly increases the permeability of HBE cell monolayers *in vitro* and the expression of VEGF, and that VEGF partially mediates the regulation of epithelial cell permeability. These *in vitro* mechanistic studies provide insights into the effects of TDI on the respiratory tract.

## Materials and methods

### *Preparation of toluene diisocyanate–human serum albumin conjugates*

TDI–HSA conjugates were prepared by a modification of Son's method [17]. Briefly, 200 µL TDI (2,4 TDI: 80%; 2,6 TDI: 20%) was added to 9 mL of 1% HSA in phosphate-

buffered saline with constant stirring. Ammonium carbonate (2 M) was added to each aliquot to terminate the reactions. All reacted samples including control HSA were centrifuged at 3000 g for 20 min at room temperature to remove unreacted TDI. The product was then dialysed using cellulose membranes (molecular weight cut-off 12 000, D9777, Sigma Chemical Co., St. Louis, MO, USA) for 3 days against 4 L of 0.1 M ammonium carbonate with an equal volume of 20% trichloroacetic acid. The dialysed product was redissolved in 1 M sodium hydroxide, and then dialysed overnight three times with 4 L of distilled water.

### *Determination of the amount of toluene diisocyanate bound to human serum albumin*

The degree of substitution was determined by a modification of Son et al.'s assay [17]. The assay was performed using 50 µL aliquots of sample, to which 0.6 mL of concentrated HCl, 0.1 mL of distilled water, and 0.2 mL of 0.029 M sodium nitrate were added. After 5 min of reaction, 0.6 mL of 0.031 M sodium 2-naphthol-3,6-disulphonate in 7.4 M ammonium hydroxide was added. Each assay sample was mixed thoroughly, allowed to stand for 5 min, and read spectrophotometrically at 500 nm. The amount of isocyanate bound to HSA was measured using a standard calibration curve constructed from different concentrations of *p*-toluidine according to a modified Gutmann assay [18]. After acquiring the equation of linear regression, 0.4 mL TDI–HSA and HSA, respectively, were measured with the same process. The HSA content of a conjugated sample was determined by the Lowry method using bovine serum albumin as a standard.

### *Native gel analysis*

Proteins were mixed with 10% glycerol in gel running buffer and then subjected to electrophoresis on 10% native polyacrylamide gels, pH 8.3, as described previously [17, 19]. Gels were stained with Coomassie brilliant blue G250.

### *Preparation of cells*

HBE135-E6E7 cells, an HBE cell line (obtained from ATCC, Rockville, IN, USA, CRL-2741<sup>TM</sup>) was maintained in growth medium consisting of RPMI1640 medium supplemented with 15% heat-inactivated fetal calf serum (Hangzhou Sijiqing Bioengineering Material Research Institute, Hangzhou, China) containing penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively). The plates were then incubated at 37 °C in a 5% CO<sub>2</sub> incubator. After cells reached complete confluence, they were seeded into six-well or 24-well plates at a density of  $2.5 \times 10^4$  cells/cm<sup>2</sup>. When the cells reached 90% confluence, the medium was

changed to keratinocyte serum-free medium (Gibco, NY, USA) and treated with 40 µg/mL TDI-HSA and 40 µg/mL HSA. After a 24-h incubation at 37 °C, 5% CO<sub>2</sub>, the culture supernatants were collected for ELISA analysis, and HBE cells were harvested with Trizol reagent (Invitrogen, Carlsbad, CA, USA).

#### Permeability measurements

Permeability studies were carried out using Man's method with minor modifications [20]. Briefly, HBE135-E6E7 cells were seeded at a density of  $1 \times 10^5$  cells/cm<sup>2</sup> on gelatin-coated costar Transwell Clear membranes (6.5 mm diameter, 0.4 µm, pore size, Sigma Chemical Co.) and allowed to grow for 1–2 days until complete confluence was reached. The medium was then changed to keratinocyte-serum-free medium and cells were treated with 40 µg/mL TDI-HSA or 40 µg/mL HSA for 24 h. Then, 200 µL phenol red-free RPMI-1640 containing 0.5 mg/mL fluorescein isothiocyanate (FITC)-labelled dextran (Sigma Chemical Co.; molecular weight 77 000) was added to the apical compartments (luminal side). Then, 600 µL phenol red-free RPMI-1640 without FITC-labelled dextran was added to the basal compartments (non-luminal side), and the plates were incubated at 37 °C for 90 min. Samples from the apical and basal compartments were analysed by fluorimetry. Excitation and emission wavelengths were 485 and 535 nm, respectively. The measured values were used in the following equation to determine the permeability coefficient of dextran ( $P_d$ ):

$$P_d = [A_1]/t \times 1/A_2 \times v/[L]$$

where  $[A_1]$  is the concentration of dextran in the basal compartment;  $t$  the time (s);  $A_2$  the area of the filter membrane (cm<sup>2</sup>);  $V$  the volume of liquid in the basal compartment (cm<sup>3</sup>); and  $[L]$  the concentration of dextran in the apical compartment. The results are expressed as  $[P_d]/[\text{control } P_d] \times 100\%$ .

#### Reverse transcriptase-polymerase chain reaction

Total RNA from control, TDI-HSA, and HSA-activated HBE135-E6E7s cells were isolated with Trizol kits (Invitrogen), according to the manufacturer's instructions. The primers (Invitrogen, Shanghai, China) were: 5'-TTGCTGCTCTACCTCCAC-3' (sense) and 5'-AAATGCTTTCTCCGCTCT-3' (antisense) for VEGF (GenBank accession no. NM001025369), and 5'-GTGGACATCCGCAAAGAC-3' (sense) and 5'-GAAAGGGTGTAAACGCAACT-3' (antisense) for β-actin. RT-PCR was performed as recommended in the Takara one-step RNA PCR kit (AMV) (Takara, Shiga, Japan). Samples were amplified in a thermal cycler (GeneAmp PCR system 2400 PCR, Perkin-Elmer Cetus, Hayward, CA, USA). Reverse transcription was performed at 50 °C for 45 min, followed by 30 cycles. Each cycle

included denaturation at 95 °C for 30 s, primer annealing for VEGF at 57.2 °C for 60 s, and extension at 72 °C for 60 s. Amplification products were analysed by agarose gel electrophoresis and images were captured on the DOC GEL 2000 gel imaging system (Bio-Rad, CA, USA).

#### Enzyme-linked immunosorbent assay

VEGF levels in TDI-treated HBE culture supernatants were measured using a sandwich enzyme immunoassay (Jing-Mei Biotech, Shenzhen, China) using ELISA kits according to the procedure recommended by the manufacturer. Briefly, the samples were added to 96-well microtitre plates, previously coated with a murine monoclonal antibody to VEGF. The unbound protein was washed three times, and an enzyme-linked polyclonal antibody specific to VEGF was added. The plate was washed three times, and a substrate solution was added to the wells. After 30 min of incubation, a stop solution was added to each well. The amount of VEGF was determined by the optical density of the samples at 450 nm using an ELISA reader.

#### Effect of vascular endothelial growth factor-neutralizing antibody on the permeability of HBE135-E6E7 cell monolayers

HBE135-E6E7 cell cultures were treated with either 40 µg/mL HSA, 40 µg/mL TDI-HSA or 40 µg/mL TDI-HSA plus 0.02 µg/mL VEGF-neutralizing antibody (R&D Co., Minneapolis, MN, USA). The concentration of the neutralizing antibody chosen for the experiment was based on kit instructions and preliminary experiments. In brief, according to the kit, if VEGF is present at concentrations < 10 ng/mL, 0.01–0.03 µg/mL of neutralizing antibody is sufficient to neutralize VEGF. Preliminary experiments showed that VEGF levels in the supernatant almost reached zero when 0.01 µg/mL neutralizing antibody was added to the supernatant with the highest concentration. Thus, we chose to use 0.02 µg/mL of neutralizing antibody as the standard treatment concentration.

#### Transmission electron microscopy

HBE135-E6E7 cells were seeded in 25 cm<sup>2</sup> culture flasks until complete confluence was achieved. Cells were then treated with 40 µg/mL HSA, 40 µg/mL TDI-HSA, and 40 µg/mL TDI-HSA+VEGF-neutralizing antibody. Normal HBE135-E6E7 cells were used as a control. Specimens prepared for TEM were treated first with 5% glutaraldehyde solution for 60 min, then with 1% osmium tetroxide, and dehydrated in a standard ethanol series before embedding them in Epon acrylic resin and sectioning as described previously [21]. Then, the specimens were cut into smaller pieces. Sections were viewed on a Hitachi H7500 TEM (Hitachi, Ibaraki, Japan) and images were

captured digitally. Ultrastructure observations were made from multiple sites (> 10) of junctional complexes that were clearly identified. Three separate TEM experiments were performed. Quantitative analysis of the TEM data was performed by scoring the number of TJs in approximately 30 randomly selected cell–cell contact sites in each experiment. The following criteria were used for estimating TJs in junctional complexes [22]: (a) a normal TJ was defined as a cell–cell junction with at least two ‘kissing’ points, and (b) the disruption of each junction was defined as a wider intracellular space. According to these criteria, the numbers of normal TJs was counted by three individuals in a blind manner, and then the percentages of normal TJs were derived for each specimen.

### Statistical analysis

Data are expressed as mean ± standard error of the mean and analysed by one-way ANOVA using SPSS version 11.5. The LSD method was used to determine the differences between all the experimental groups. The  $\chi^2$ -test was used to compare the percentages of TJs in the different groups.  $P < 0.05$  was considered statistically significant.

## Results

### Preparation and determination of toluene diisocyanate–human serum albumin conjugates

TDI–HSA conjugates were analysed on native gels. Mock HSA was prepared and used as a control. Biophysical/biochemical changes in TDI–albumin conjugates were evidenced by a change in migration during native gel electrophoresis (Fig. 1). The migration of TDI–HSA conjugates differed considerably from that of mock HSA. Because TDI–HSA is not stable, the concentration of TDI–HSA was determined before the experiment. After reaction with sodium 2-naphthol-3,6-disulphonate,

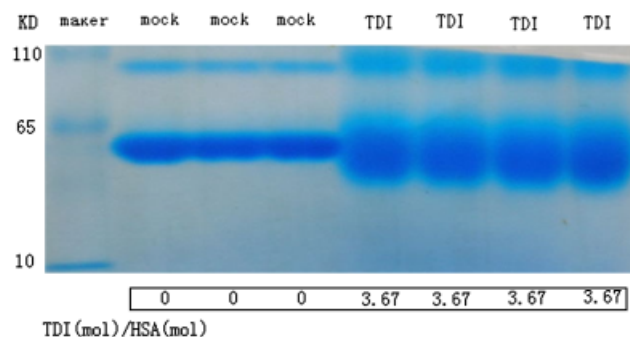


Fig. 1. Native gel analysis of toluene diisocyanate (TDI)–albumin conjugates prepared by liquid-phase TDI exposure. Albumin exposed to air (mock), isocyanate was electrophoresed under native conditions. TDI represents an 80/20 ratio of 2,4/2,6-TDI, whereas mock represent untreated human serum albumin (HSA).

TDI–HSA conjugates turned into jacinth, while the HSA and the control group turned into pallide-flavens (Fig. 2). TDI concentrations were calculated by the equation of linear regression generated from different concentrations of *p*-toluidine: ( $y = -1.853 + 27.831x$ ). HSA concentrations were calculated based on the Lowry assay: ( $y = -6.190 + 91.713x$ ). The concentrations of TDI and HSA were 18.65 and 1938 µg/mL, respectively. The molar ratio of TDI to HSA was 3.67.

### Toluene diisocyanate–human serum albumin increases HBE135–E6E7 permeability

FITC-labelled dextran was used to evaluate the effects of TDI–HSA or TDI–HSA on HBE135–E6E7 cell monolayer permeability. TDI–HSA significantly increased HBE135–E6E7 cell monolayer permeability ( $n = 6$ ,  $P < 0.01$ ), whereas there was no significant difference between the control and HSA groups (Table 1).

### Toluene diisocyanate induces vascular endothelial growth factor expression in HBE135–E6E7 cells

Cells treated with TDI–HSA had significantly higher VEGF165 to  $\beta$ -actin and VEGF189 to  $\beta$ -actin ratios compared with control and HSA-treated cells ( $P < 0.01$ ;  $n = 4$ ; Table 2 and Fig. 3).

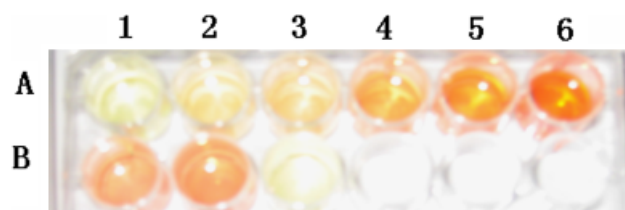


Fig. 2. Determination of the amount of toluene diisocyanate (TDI) bound to human serum albumin (HSA). A1–A6 indicate control, 20%, 25%, 40%, 80%, and 100% *p*-toluidine to distilled water (V/V), respectively. B1–B2 indicate TDI–HSA and B3 indicates the HSA group.

Table 1. Permeability coefficient changes of epithelial monolayers among groups

| Groups | N | Permeability coefficients      |
|--------|---|--------------------------------|
| A      | 6 | 100.00% ± 33.51%               |
| B      | 6 | 104.98% ± 34.68%               |
| C      | 6 | 287.36% ± 20.61%* <sup>#</sup> |
| D      | 6 | 206.34% ± 37.26% <sup>Δ</sup>  |

Note: A, control; B, 40 µg/mL HSA; C, 40 µg/mL TDI–HSA; D, 40 µg/mL TDI–HSA + 0.2 µg/mL VEGF-neutralizing antibody.

\* $P < 0.01$  vs. control.

<sup>#</sup> $P < 0.01$  vs. B.

<sup>Δ</sup> $P < 0.01$  vs. A, B, C groups.

HSA, human serum albumin; TDI, toluene diisocyanate; VEGF, vascular endothelial growth factor.

**Table 2.** Comparison of vascular endothelial growth factor/ $\beta$ -actin ratio of HBE135-E6E7 of different groups

| Groups | N | VEGF189/ $\beta$ -actin ratio     | VEGF165/ $\beta$ -actin ratio     |
|--------|---|-----------------------------------|-----------------------------------|
| A      | 4 | 0.1868 $\pm$ 0.0088               | 0.3486 $\pm$ 0.0286               |
| B      | 4 | 0.2058 $\pm$ 0.0228               | 0.3494 $\pm$ 0.0443               |
| C      | 4 | 0.3232 $\pm$ 0.0160 <sup>*#</sup> | 0.7496 $\pm$ 0.0346 <sup>*#</sup> |

Note: A, control; B, 40  $\mu$ g/mL HSA; C, 40  $\mu$ g/mL TDI-HSA.

<sup>\*</sup> $P$ <0.01 vs. A group.

<sup>#</sup> $P$ <0.01 vs. B group.

HSA, human serum albumin; TDI, toluene diisocyanate; VEGF, vascular endothelial growth factor.

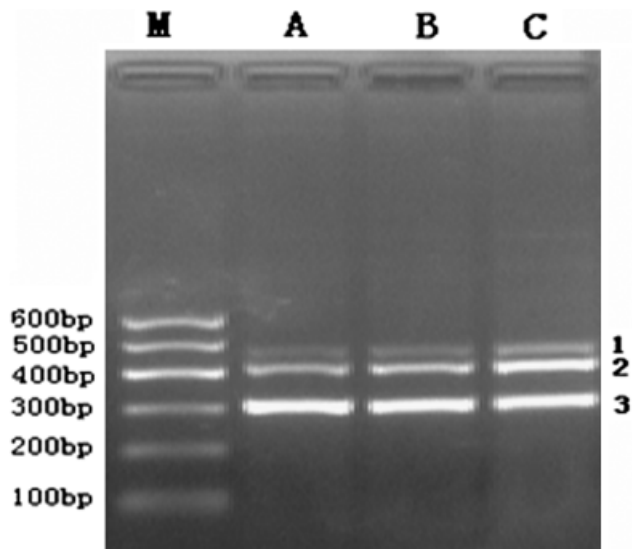


**Fig. 3.** Toluene diisocyanate (TDI)-induced vascular endothelial growth factor (VEGF) mRNA expression in HBE135-E6E7. Lane M indicates molecular weight markers; lanes A, B, and C indicate control, 40  $\mu$ g/mL HSA, and 40  $\mu$ g/mL TDI-HSA, respectively.  $\beta$ -actin served as a positive housekeeping gene. 1, 2, 3 indicates VEGF189, VEGF165, and  $\beta$ -actin, respectively.

#### *Toluene diisocyanate induces vascular endothelial growth factor release by HBE135-E6E7 cells*

TDI-HSA-treated cells had significantly higher VEGF concentrations in the cell culture than control or HSA-treated cells (3418 $\pm$ 339 vs. 892 $\pm$ 241, and 888 $\pm$ 231 pg/mL, respectively;  $P$ <0.01,  $n$  = 7; Fig. 4). These results showed that TDI-HSA induced VEGF release from HBE135-E6E7 cells.

#### *Vascular endothelial growth factor-neutralizing antibody partially but significantly decreased toluene diisocyanate-human serum albumin-induced HBE135-E6E7 monolayer permeability*

After adding VEGF-neutralizing antibody to TDI-HSA-treated cells, the permeability coefficients significantly decreased ( $P$ <0.01,  $n$  = 6), but there was still a significant

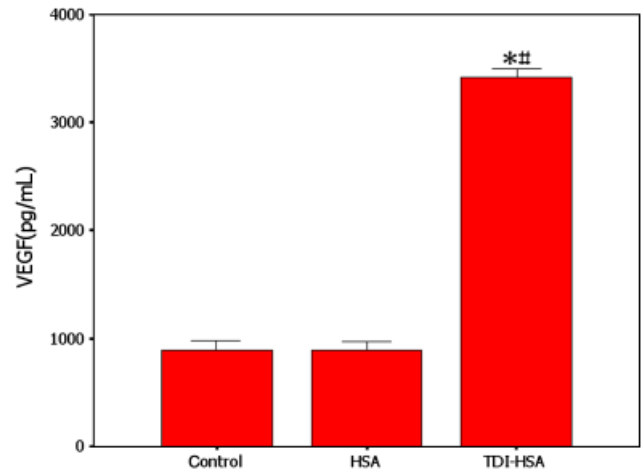


**Fig. 4.** Toluene diisocyanate (TDI) induces vascular endothelial growth factor (VEGF) release from HBE135-E6E7 cells. Data represent mean $\pm$ SE. <sup>\*</sup>, <sup>#</sup>Statistical significance ( $P$ <0.01,  $N$  = 7) compared with control cultures and with the human serum albumin (HSA) group.

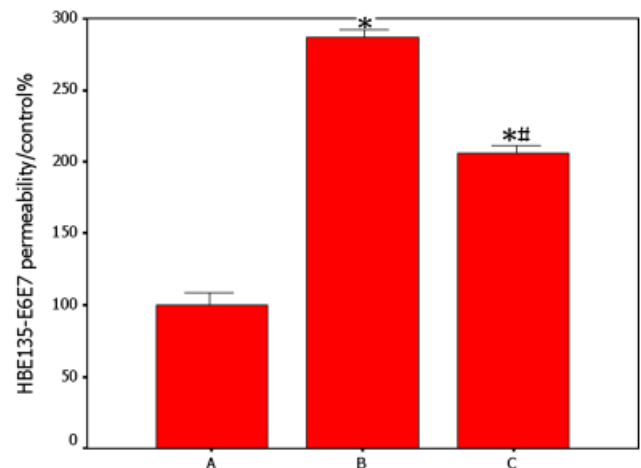


**Fig. 5.** Vascular endothelial growth factor (VEGF)-neutralizing antibody partially but significantly decreased toluene diisocyanate (TDI)-induced HBE135-E6E7 cell permeability. (a), (b), and (c) indicate control, 40  $\mu$ g/mL TDI-HSA, and 40  $\mu$ g/mL TDI-HSA+0.02  $\mu$ g/mL VEGF-neutralizing antibody, respectively. Data represent mean $\pm$ SE. <sup>\*</sup>Statistical significance ( $P$ <0.01,  $N$  = 6) compared with control cultures. <sup>#</sup>Statistical significance ( $P$ <0.01,  $N$  = 6) compared with TDI-HSA. HSA, human serum albumin.

difference in comparison with the control group ( $P$ <0.01,  $n$  = 6; Table 1, Fig. 5). These results suggest that VEGF partially mediates the modulation of HBE135-E6E7 monolayer permeability by TDI-HSA.

#### *Toluene diisocyanate-human serum albumin significantly decreases tight junction numbers in confluent HBE135-E6E7 cells, and neutralizing vascular endothelial growth factor antibody did not prevent this effect*

In normal resting HBE135-E6E7 cells and HSA-treated cells, cell-cell junctions were very tight. After treatment

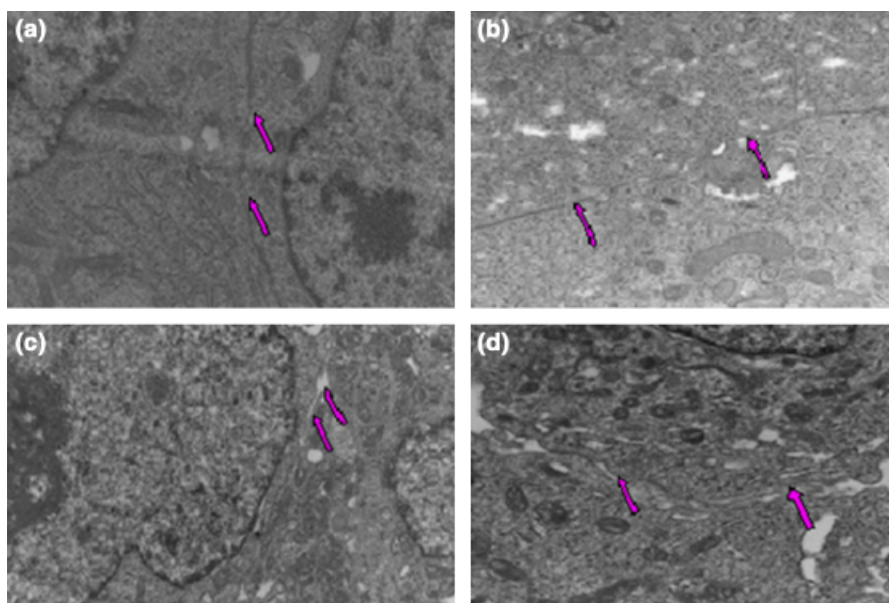


Fig. 6. High magnification ( $\times 25\,000$ ) of HBE135-E6E7 cells exposed to toluene diisocyanate (TDI). (a), (b), (c), and (d) indicate the control, human serum albumin (HSA) treatment group, TDI-HSA treatment group, and TDI-HSA + 0.02  $\mu\text{g}/\text{mL}$  VEGF-neutralizing antibody, respectively. Arrows in (a) and (b) denote tight junctions. Arrows in (c) and (d) denote widening of cell junctions.

with TDI-HSA, cell-cell junctions became relaxed. There were fewer TJs, and inclusion of neutralizing VEGF antibody did not restore the original number of TJs (Fig. 6).

Quantitative analysis of TJs showed that control cells and HSA-treated cells had TJs in 84.4% and 82.4% of cell-cell contacts, respectively. In contrast, TDI-HSA-treated cells showed putative TJs in 20.0% of the cell-cell contacts ( $P < 0.001$ ). However, there were no significant differences between cells treated with TDI-HSA + 0.02  $\mu\text{g}/\text{mL}$  of VEGF-neutralizing antibody (22.2%) and cells treated with TDI-HSA ( $P > 0.05$ ). Taken together, these results further supported the idea that TDI increases HBE135-E6E7 cell permeability, and that the VEGF pathway may be one of the major pathways mediating the regulation of HBE135-E6E7 cell monolayer permeability by TDI-HSA.

## Discussion

In the current study, we primarily focused on HBE135-E6E7 responses to TDI-HSA *in vitro*. In a previous study [23], to establish appropriate TDI exposure levels following TDI-HSA and HSA exposure, cell viability was assessed by the methyltetrazolium method. Those results showed that cell viability decreased or increased in a dose-dependent manner and that this effect was statistically significant ( $P < 0.05$ ) at 100  $\mu\text{g}/\text{mL}$  TDI-HSA or 60  $\mu\text{g}/\text{mL}$  HSA. Hence, we chose 40  $\mu\text{g}/\text{mL}$  HSA and 40  $\mu\text{g}/\text{mL}$  TDI-HSA in subsequent studies.

Our results demonstrate that, in comparison with 40  $\mu\text{g}/\text{mL}$  HSA or no treatment, treatment with 40  $\mu\text{g}/\text{mL}$

TDI-HSA for 24 h significantly increases cell permeability, which indicates that this effect on permeability results from exposure to TDI. The effect of TDI-HSA on cell membrane integrity was also examined using TEM. The results showed that TDI considerably reduces the number of TJs in HBE135-E6E7 cells compared with HSA-treated and control cells. This further supported the idea that TDI increases HBE135-E6E7 permeability.

As part of an evaluation of the integrity of HBE monolayers following TDI exposure, Lange *et al.* [8] reported that transepithelial resistance remained unchanged following a 30-min exposure to TDI concentrations of 100 p.p.b. or lower. This concentration of TDI also did not significantly change cell viability. These observations indicate that, at these concentrations, TJs and apical membranes remain intact. HBE cells exposed to 100–500 p.p.b. TDI vapour showed increasing pyknosis. Higher magnification further showed disorganization of tight and gap junctions. At 1000 p.p.b. TDI, transepithelial resistance was almost lost. Hence, our results are not consistent with Lange's reports. The present study supports the idea that TDI increases cell permeability without a significant influence on cell viability. The difference may depend on the following factors: (i) Lange *et al.* [8] chose TDI vapour as a stimulator, which may be different from TDI-HSA; also we have not calculated the equivalency between TDI vapour and TDI-HSA; (ii) the observation point was 30 min after 20–100 p.p.b. TDI stimulation, which may not be long enough to stimulate cytokine release, a mechanism that regulates immunity and permeability [9, 24, 25]. In our experiments,

stimulation lasted for 24 h, at which time many cytokines associated with immunity and inflammation are released [9, 24, 25] and mediate cell permeability.

The mechanisms associated with TDI-induced increases in cell permeability remain unclear, especially the conditions under which TDI affects cell viability. The sites of permeability regulation include transmembrane cell passageways and intercellular channels. The latter is the major pathway for the transport of water, electrolytes, and macromolecules. It is also the primary mechanism for a change in epithelial permeability under pathological conditions. The dynamic balance in the regulation of intercellular channels is decided by the conjunction of intercellular, cell-interstitial substances, and concentric forces produced by self-contraction of epithelial cells. The reduced expression or abnormal distribution of cell-cell junction proteins, such as occludin and ZO-1, and alterations of the cytoskeleton may also increase cell permeability [26].

Antony et al. [25] reported that cockroach extract antigen (CrAg) increases the permeability of bronchial epithelium indirectly through the induction of the angiogenic cytokine VEGF, mainly known for its potential to induce vascular permeability. Also, bronchial epithelium, mesothelial cells, and retinal epithelium can produce VEGF under the stimulation of viral and bacterial cytokines [25, 27–30]. VEGF can down-regulate the expression of TJ proteins such as occludin and ZO-1, which then changes the distribution of the proteins in and out of the cell, and increases permeability by widening spaces between cells [31]. Sun et al. [32] reported that VEGF may induce the phosphorylation of myoglobin, a cytoskeleton component, thereby resulting in contraction of actin and increasing cell permeability. All these studies suggest that VEGF plays an important role in the regulation of cell permeability. Thus, we were very interested in determining whether the increase in HBE permeability induced by TDI was mediated by the VEGF pathway.

In a previous study, we showed that TDI-HSA increases VEGF expression in HBE135-E6E7 cells in a dose-dependent manner [23]. The present study further shows that TDI-HSA increases the expression of VEGF at the protein level. To evaluate whether VEGF mediates the increase in cell permeability induced by TDI-HSA, we used a VEGF-neutralizing antibody to block the VEGF pathway. The results showed that the neutralizing antibody partially inhibited TDI-HSA-mediated increase in HBE135-E6E7 cell permeability. Using TEM, we found that cells treated with TDI-HSA had fewer TJs than control and HSA-treated cells, and addition of the anti-VEGF antibody did not restore the original number of TJs. The effects of the anti-VEGF-neutralizing antibody in this study can be explained as follows: first, the results suggest that other pathways (besides the VEGF pathway) are implicated in the alteration of permeability in HBE135-E6E7 cells exposed to TDI-HSA; this will need further experimental

evidence. Compared with Antony et al.'s [25] investigation, in which neutralizing antibodies to VEGF almost completely restored AEC permeability in CrAg-treated cells to normal levels, in our study, the same treatment only restored approximately 30% of cell permeability after treatment with TDI-HSA. This result indicates that TDI and CrAg may change the permeability of bronchial epithelial cell through different mechanisms or different pathways. Second, previous studies have suggested that TDI-induced increases in cell permeability are involved in the dysfunction of TJ proteins and contraction of the cytoskeleton [8, 24]. Our results show that VEGF may participate in the contraction of the cytoskeleton, but it remains uncertain as to whether VEGF regulates the expression of TJ proteins such as occludin and ZO-1 [31, 32]. Also, TEM often gives results partly inconsistent with the FITC-dextran method, which may be because the former is always used to determine transient structural deformations, while the latter is more accurate in the quantitative analysis of cell permeability [8, 20].

Our results did not show the pathological significance of VEGF increase or the mechanisms underlying the increased expression of VEGF in HBE135-E6E7 cells exposed to TDI. Studies have shown that VEGF activates dendritic cells and these cells subsequently promote antigen sensitization and T cell polarization to the Th2 phenotype [10, 11]. Notably, VEGF also induces a variety of pro-inflammatory changes in the allergic lung, including leucocyte accumulation, collagen deposition, myocyte hyperplasia, goblet cell metaplasia, and blood vessel alterations. In other studies, we showed that TDI-HSA increases VEGF expression partly through the phosphatidylinositol-3-kinase (PI<sub>3</sub>K) pathway, and that dexamethasone has no effect on VEGF expression induced by TDI-HSA (unpublished data).

In the present study, we demonstrated for the first time that (i) TDI increases epithelial cell permeability without reducing cell viability; (ii) TDI-HSA causes VEGF release in HBE135-E6E7 cells; and (iii) VEGF partly mediates the increase in monolayer permeability *in vitro*. However, because of the difference between epithelial cells *in vitro* and *in vivo*, the results of this research may not be identical to the clinical situation. Therefore, the role of TDI and the mechanisms by which it modulates epithelial permeability in airway diseases need further study. We intend to further answer these questions by using samples from an animal model or from patients with TDI-induced asthma in the near future.

In summary, TDI-induced increase in HBE cell monolayer permeability is partially mediated through the VEGF pathway. These findings suggest not only that VEGF released from HBE cells may play an important role in TDI-induced asthma but also that another important pathway exists that mediates the TDI-induced increase in HBE cell monolayer permeability.

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