

Effect of PM2.5 mediated oxidative stress on the innate immune cellular response of Der p1 treated human bronchial epithelial cells

Z.-J. BAO, Y.-M. FAN, Y.-F. CUI, Y.-F. SHENG, M. ZHU

Tuberculosis Department of Hangzhou RedCross Hospital, Hangzhou City, Zhejiang, China

Abstract. – OBJECTIVE: To investigate the effect of stimulation of Human Bronchial Epithelial Cells (HBEC) by Der p1 and PM2.5 on the expression of innate immune cell factors to find new therapeutic targets for treatment of bronchial asthma.

MATERIALS AND METHODS: The Der p1 antigen exposure model in the HBEC line, 16HBE-14o, was established *in vitro*. PM2.5 at a concentration of 50 $\mu\text{M}/\text{cm}^2$, was added to these cells for 0.5 h, 1 h, 2 h and 3 h. Cells were treated with the following reagents for the indicated times: 300 ng/mL Der p1 for 21 h, 50 $\mu\text{M}/\text{cm}^2$ PM2.5 for 3 h, 10 mM Nac for 3 h and PM2.5 contamination for 3 h. The experiment was divided into five groups: control (group A), Der p1 exposure group (group B), PM2.5 treated group (group C), PM2.5+Der p1 exposure group (group D), Nac+PM2.5+Der p1 exposure group (group E). ELISA method was adopted to test the expression levels of malondialdehyde, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP), and Real-time RT-PCT was used to measure IL-25, IL-33 and TSLP mRNA.

RESULTS: The protein and mRNA levels of malondialdehyde, IL-25, IL-33 and TSLP in group D were significantly higher than those in the other groups, while the protein and mRNA levels of malondialdehyde, IL-25, IL-33 and TSLP in group E were significantly lower than those in group D ($p < 0.05$).

CONCLUSIONS: PM2.5 can enhance the Der p1 antigen-induced HBEC innate immune response through the expression of IL-25, IL-33 and TSLP, which may exacerbate the occurrence rate of bronchial asthma.

Key Words:

PM2.5, Oxidative stress, Der p1 antigen, HBEC, Innate immune, Cell factor, Bronchial asthma, TSLP, Nac.

Introduction

Bronchial asthma is a common disease, which occurs worldwide, and the incidence rate is increasing annually¹. With the increase in air pollution

in recent years, the quality of life of patients is being affected. Additionally, the risks of chronic obstructive pulmonary disease and chronic respiratory failure, which may result in acute death, are increased². The pathogenesis of bronchial asthma is complex, and the majority of scholars believe the main pathological and physiological features³ of bronchial asthma include chronic airway inflammation, bronchial hyper-responsiveness (BHR) and reversible airflow obstruction. Airflow obstruction in turn, may result in different degrees of airway remodeling and formation of new and inordinate airway tissues as the disease progresses, thus aggravating the conditions of airflow obstruction. PM2.5 is a fine particulate matter and accounts for 70% of inhalable particles⁴. In areas with heavy air pollution, the composition of various poisonous gases and exposure of allergens are two major chronic pathogenic factors⁵, which results in (and worsen) the symptoms of bronchial asthma. The exposure of house dust mite antigens can affect asthma innate immune response through up-regulation of IL-7 like cytokines in airway epithelium, most notably thymic stromal lymphopoietin (TSLP), IL-25 and IL-33⁶. There are few reports on the specific impact of exposure to PM2.5 and co-exposure of PM2.5 and house dust mite antigen on the response of airway epithelial cells.

Materials and Methods

Cells

The human bronchial epithelial cell line, 16HBE-14o, was purchased from the Cell Laboratory of the UK's University of Southampton, and the cells were conventionally cultured in MEM-10 cell culture medium containing 10% of fetal calf serum (FCS). Cells were maintained in media and digested by trypsin for twice weekly passaging.

A total of 5×10^4 cells were seeded in each well of a 24-well tissue culture plate, and the MEM-10 cell culture medium was replaced by serum-free MEM after cells reached 90% confluency. Cells were then placed and cultured in the incubator at 37°C and 5% CO₂, overnight until the cells stopped growing.

Experimental Groupings

The experiment was divided into five groups: the control group (group A), Der p1 treated group (group B), PM2.5 treated group (group C), PM2.5 + Der p1 treated group (group D), and Nac+PM2.5+Der p1 treated group (group E). Sixteen wells were made available for each group in 96-well compound plates, and the cellular concentration for each well was 5×10^4 cells/well. The method as described by Rusznak et al⁷ was adopted for group B, namely that 16HBE-14o cells under growth arrest were treated with 300 ng/mL Der p1 for 24 h. The method adopted in-group C was as follows: PM 2.5 at a concentration of 50 $\mu\text{M}/\text{cm}^2$ was added to 16HE-14o cells for 0.5 h, 1 h, 2 h and 3 h. The method adopted in group D was as follows: 16HBE-14o cells were treated with 300 ng/mL Der p1 for 21 h and treated with PM2.5 at a concentration of 50 $\mu\text{M}/\text{cm}^2$ for 3 h. The method adopted in-group E was as follows: cells were treated with 10 mM acetylcysteine (Nac) and PM2.5 simultaneously for 3 h.

ELISA and RT-PCR

ELISA method was chosen to test the expression levels of malondialdehyde, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) after cells were cultured for 3 h, 6 h, 12 h and 24 h respectively. 16 human bronchial epithelial cell line-(16HBE)-14o cell line supernatant was collected and stored at -80°C until use after being centrifuged at low temperature. A total of 1 ml cell culture supernatant was collected and stored. The ELISA kit was from the Nanjing Jiancheng Bioengineering Institute and used according to the manufacturer's instructions. Real-time RT-PCR was adopted to measure IL-25, IL-33 and TSLP mRNA and a semi-quantitative analysis was carried out. The cells were washed with phosphate-buffered saline (PBS) at 4°C, harvested, and treated with Trizol. They were then filtered after the cell culture supernatant was collected. The cells went through the process of biphasic separation, RNA precipitation, washes, redissolution, determination of concentration by ultraviolet spectrophotometry, and synthesis of cDNA. The

samples were added to the PCR reaction system, after which semi-quantitative analysis was carried out according to Takahashi et al⁸. Results were calculated according to the $^{-2\Delta\text{Ct}}$ method.

Statistical Analysis

All data were processed using SPSS19.0 statistical software (SPSS Inc., Chicago, IL, USA). Measurement data are expressed as mean $\pm$ standard deviation, comparisons among groups were analyzed by single factor ANOVA, and intra-group comparisons were by repeated measurement data. $p < 0.05$ was taken as statistically significant.

Results

Comparison of expression levels of malondialdehyde, IL-25, IL-33 and TSLP

There were no significant changes in the levels of malondialdehyde, IL-25, IL-33 or TSLP in-group A at any of the time points. The expression levels of malondialdehyde, IL-25 and IL-33 reached peak levels in group B at 6 h, and the expression levels of malondialdehyde and TSLP continued to increase over time. The levels of malondialdehyde and cytokines increased over time in-group C. The levels of malondialdehyde and IL-25 were equal to those of IL-33 after 12 h and 24 h of culture. The difference was not statistically significant when malondialdehyde and TSLP in-group C were compared with that in-group B at each time point. In-group D, the levels of malondialdehyde and cytokines increased over time. Malondialdehyde content and the levels of the different cytokines in-group D at each time point were higher than the other groups. Furthermore, the levels of malondialdehyde and cytokines in-group E at each time point were significantly lower than in-group B, C and D ($p < 0.05$) (Figures 1-3).

Malondialdehyde content increased over time in-group B, C and D, and malondialdehyde content in-group D was significantly higher than in-group B. Malondialdehyde content in-group B was significantly higher than in-group C, and the difference had statistical significance ($p < 0.05$). Malondialdehyde content in group E at each time point was significantly lower than in group B, C and D, and there was no time-dependent effect in group E (Figure 4).

Comparison of Cytokine mRNA Levels

According to the intra-group comparison of IL-25, IL-33 and TSLP mRNA in each group, at

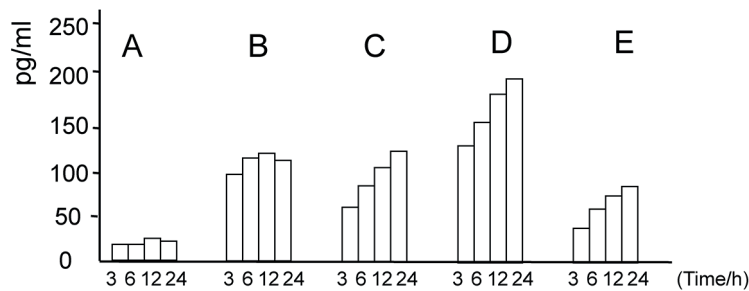


Figure 1. Expression level of IL-25 in each group at each time point (Group A: single airway epithelium; Group B: airway epithelium + Der p1; Group C: airway epithelium + PM2.5; Group D: airway epithelium + Der p1 + PM2.5; Group E: Nac + airway epithelium + Der p1 + PM2.5).

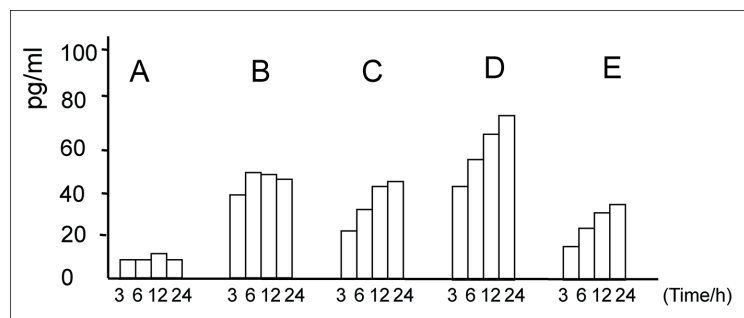
each time point, there were no statistically significant differences ($p > 0.5$). According to the comparison of IL-25, IL-33 and TSLP mRNA among the different groups (inter-group comparisons), the expression levels of IL-25, IL-33 and TSLP mRNA in group D were significantly higher than in group B and C. Furthermore, the expression levels of IL-25, IL-33 and TSLP mRNA in group E were significantly lower than in group B, C and D ($p < 0.05$) (Table I).

Discussion

At present, it is thought that the essential feature of asthma is airway inflammation. Multiple cells play a role, including eosinophils, mast cells, lymphocytes, neutrophils, airway epithelial cells and cellular elements. The potential therapeutic targets¹¹ of asthma may include asthma-related inflammation, immune cell cytokines, chemotactic factors, transcription factors and enzymes. According to recent studies¹², the Th1/Th2 balance can be shifted toward Th2¹³ by upregulating Th2 cell expression. IL-25 and IL-33 are newly discovered Th2 cytokines, and IL-25 and IL-33 can participate in the Th2-type immune response¹⁴ through the coordinate induction of IL-4 and IL-13 Th2 cytokines. There are large differences among the chemical components of PM2.5

because of differences in region and sources of pollution. However, the chemical components of PM2.5 are mainly fine particles and air pollution components enriched with the aforementioned fine particles, including sulfur dioxide, nitrogen dioxide, polycyclic aromatic hydrocarbons, metal aerosols, as well as others. Because of the aerodynamic features of PM2.5, airborne PM2.5 can enter the airway and remain in alveoli after inhalation. This can result in inflammation within bronchial tissues, oxidative stress injury, and can induce an intense systemic inflammatory response, causing injuries of respiratory and circulatory systems as well as others. Additionally, long-term exposure can result in cancer and deformity¹⁷. Many studies⁸ have shown that PM2.5 particles, oxynitride, polycyclic aromatic hydrocarbons, heavy metals and other harmful components, can result in the increased reactive oxide species (ROS) in the microenvironment of airways through oxidative stress. Malondialdehyde, as a biomarker of ROS in cell culture, can be measured. It can be measured in culture supernatants or lung tissues to reflect the degree of oxidative stress, as increases of ROS would cause damage to cell membranes, chromosomes and cellular components. Also, increases in ROS can activate NF- κ B signaling, and stimulate the secretion of IL-8, tumor necrosis factors and other inflammatory factors from airway epithelial cells (bronchial epithelial

Figure 2. Expression level of IL-33 in each group at each time point (Group A: single airway epithelium; Group B: airway epithelium + Der p1; Group C: airway epithelium + PM2.5; Group D: airway epithelium + Der p1 + PM2.5; Group E: Nac + airway epithelium + Der p1 + PM2.5).



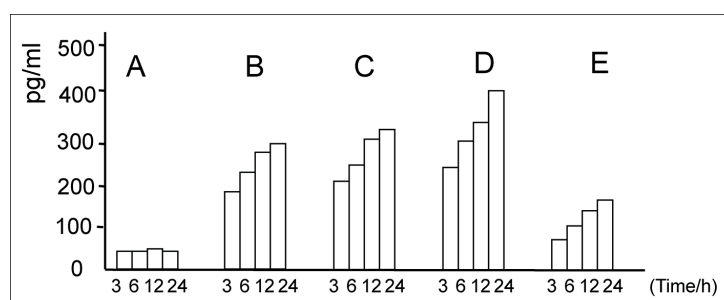


Figure 3. Expression level of TSLP in each group at each time point (Group A: single airway epithelium; Group B: airway epithelium +Der p1; Group C: airway epithelium +PM2.5; Group D: airway epithelium + Der p1+PM2.5; Group E: Nac + airway epithelium + Der p1+PM2.5).

cells or alveolar epithelial cells) and alveolar macrophages, thus resulting in increased airway and lung parenchyma aseptic inflammation as well as airway resistance. It was confirmed by viviperception and cell culture studies that the ROS quenching agent, Nac, can remove oxygen radicals and inhibit the injury related to PM2.5 oxidative stress¹⁹ by increasing the concentration of reduced glutathione. Because Nac can maintain the microenvironment where the immune cells are located, reductive and non-oxidizing, it can reduce the damage caused by oxidative stress to immune cells. Therefore, Nac is also known as an immunomodulator. Nitschke et al²⁰ showed that, the main components of air pollution, including ozone, cigarette smoke, and sulfur dioxide may increase the effects of house dust mite antigen or allergic airway inflammation stimulated by and resulting from ovalbumin, and may up-regulate the expression levels of airway epithelial IL-4, IL-5, endothelin-1 and other inflammatory factors. Also, air pollution may enhance the allergen's ability to cause disease, and there are synergistic effects existing between air pollution and an allergen's ability to cause disease. Through the present research, we can conclude that, the expression of IL-25, IL-33 and TSLP, their corresponding

mRNA levels, and malondialdehyde content in airway epithelial cells treated with PM2.5 and Der p1, are significantly higher than in the other groups. The expression levels of malondialdehyde, IL-25, IL-33 and TSLP, and their corresponding mRNA levels in the group treated with Nac, PM2.5 and Der p1, were significantly reduced, and the differences were statistically significant. It was confirmed that the co-exposure of PM2.5 and house dust mite antigen has synergetic effects on the regulation of cytokine expression levels of the innate immune response, and PM2.5 oxidative stress injury plays an important role in the process of innate immune activation and anti-free radical therapy. Nac can inhibit the effects of PM2.5 contamination in allergic airways and inhibits the production of innate immunity-related cytokines. This is helpful for physicians, to fully understand the synergistic mechanism of two major environmental factors, including allergens and air pollution in the pathogenic process of asthma, to guide the prevention and control of asthma as well as the research and development of new forms of pharmacotherapy. This also allows for deepening the understanding of the pathogenic mechanisms of PM2.5 contamination and drug prevention and control, which are important public health issues

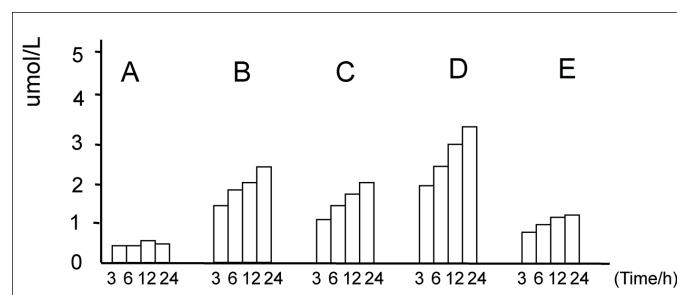


Figure 4. Comparison of Malondialdehyde in each group at each time point (Group A: single airway epithelium; Group B: airway epithelium +Der p1; Group C: airway epithelium +PM2.5; Group D: airway epithelium + Der p1+PM2.5; Group E: Nac + airway epithelium + Der p1+PM2.5).

Table 1. Comparison of cytokine mRNA levels.

Groups	IL-25 mRNA				IL-33 mRNA				TSLP mRNA			
	3h	6h	12h	24h	3h	6h	12h	24h	3h	6h	12h	24h
Group A	0.02±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	0.02±0.01	0.03±0.01	0.02±0.01	0.03±0.01	0.03±0.01	0.02±0.01
Group B	0.42±0.06	0.45±0.05	0.46±0.07	0.44±0.05	0.39±0.08	0.38±0.09	0.37±0.06	0.38±0.07	0.46±0.07	0.47±0.06	0.46±0.08	0.47±0.09
Group C	0.58±0.05	0.56±0.06	0.55±0.08	0.57±0.07	0.54±0.06	0.55p CN7	0.53±0.05	0.55±0.08	0.62±0.07	0.63±0.06	0.61±0.08	0.62±0.05
Group D	0.36±0.04	0.35±0.05	0.36±0.03	0.37±0.04	0.31±0.06	0.30±0.07	0.32±0.08	0.32±0.08	0.39±0.03	0.37±0.04	0.38±0.05	0.36±0.04
Group E	0.21±0.03	0.19±0.02	0.20±0.05	0.22±0.04	0.14±0.04	0.13±0.06	0.15±0.05	0.15±0.07	0.24±0.03	0.23±0.02	0.22±0.04	0.23±0.04
F	6.324	6.427	6.128	6.359	6.957	6.648	6.329	6.654	7.201	7.135	7.245	7.624
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

vital to the national economy and people's livelihoods. In future investigations, we will verify the effect of PM2.5 on the expression of innate immunity related cytokines which are regulated by airway epithelium resulting from house dust mite antigen using an animal model and human studies. We will verify the inhibitory effects of Nac and PM2.5 on the expression of innate immunity related cytokines, which are regulated by airway epithelium, resulting from house dust mite antigen by *in vitro* and *in vivo* studies.

Conclusions

PM2.5 can enhance the Der p1 antigen-induced HBEC innate immune cell response through the effect of oxidative stress, thus potentially increasing the occurrence rate of bronchial asthma.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

- HAN RF, LI HY, WANG JW, CONG XJ. Study on clinical effect and immunologic mechanism of infants capillary bronchitis secondary bronchial asthma treated with bacterial lysates Broncho-Vaxom. *Eur Rev Med Pharmacol Sci* 2016; 20: 2151-2155.
- ARPINELLI F, CARONE M, RICCARDO G, BERTOLOTI G. Health-related quality of life measurement in asthma and chronic obstructive pulmonary disease: review of the 2009-2014 literature. *Multidiscip Respir Med* 2016; 11: 5.
- ZHANG YX, LIU Y, XUE Y, YANG LY, SONG GD, ZHAO L. Correlational study on atmospheric concentrations of fine particulate matter and children cough variant asthma. *Eur Rev Med Pharmacol Sci* 2016; 20: 2650-2654.
- ZENG X, XU X, ZHENG X, REPONEN T, CHEN A, HUO X. Heavy metals in PM2.5 and in blood, and children's respiratory symptoms and asthma from an e-waste recycling area. *Environ Pollut* 2016; 210: 346-353.
- YANG A, JANSSEN NA, BRUNEKREEF B, CASSEE FR, HOEK G, GEHRING U. Children's respiratory health and oxidative potential of PM2.5: the PIAMA birth cohort study. *Occup Environ Med* 2016; 73: 154-160.
- JIANG C, FAN X, LI M, XING P, LIU X, WU Y, ZHANG M, YANG P, LIU Z. Characterization of Der f 29, a new allergen from dermatophagoides farinae. *Am J Transl Res* 2015; 7: 1303-1313.
- RUSZNAK C, SAPSFORD RJ, DEVALIA JL, JUSTIN JOHN R, HEWITT EL, LAMONT AG, WOOD AJ, SHAH SS, DAVIES

- RJ, LOZEWICZ S. Cigarette smoke potentiates house dust mite allergen-induced increase in the permeability of human bronchial epithelial cells in vitro. *Am J Respir Cell Mol Biol* 1999; 20: 1238-1250.
- 8) TAKAHASHI K, HIROSE K, KAWASHIMA S, NIWA Y, WAKASHIN H, IWATA A, TOKOYODA K, RENAULD JC, IWAMOTO I, NAKAYAMA T, NAKAJIMA H. IL-22 attenuates IL-25 production by lung epithelial cells and inhibits antigen-induced eosinophilic airway inflammation. *J Allergy Clin Immunol* 2011; 128: 1067-1076.
- 9) BYRNE SN, BEAUGIE C, O'SULLIVAN C, LEIGHTON S, HALIDAY GM. The immune-modulating cytokine and endogenous alarmin interleukin-33 is upregulated in skin exposed to inflammatory UVB radiation. *Am J Pathol* 2011; 179: 211-222.
- 10) NONAKA M, FUKUMOTO A, OGIHARA N, SAKANUSHI A, PAWANKAR R, YAGI T. Synergistic induction of thymic stromal lymphopoietin by tumor necrosis factor alpha and Th2 cytokine in nasal polyp fibroblasts. *Am J Rhinol Allergy* 2010; 24: e14-18.
- 11) KIM HY, UMETSU DT, DEKRUYFF RH. Innate lymphoid cells in asthma: will they take your breath away? *Eur J Immunol* 2016; 46: 795-806.
- 12) MAHMUTOVIC PERSSON I, AKBARSHAH H, MENZEL M, BRANDELIUS A, ULLER L. Increased expression of upstream TH2-cytokines in a mouse model of viral-induced asthma exacerbation. *J Transl Med* 2016; 14: 52.
- 13) SHIN SH, KIM YH, JIN HS, KANG SH. *Alternaria* induces production of thymic stromal lymphopoietin in nasal fibroblasts through toll-like receptor 2. *Allergy Asthma Immunol Res* 2016; 8: 63-68.
- 14) SALTER BM, OLIVERIA JP, NUSCA G, SMITH SG, TWOREK D, MITCHELL PD, WATSON RM, SEHMI R, GAUVREAU GM. IL-25 and IL-33 induce Type 2 inflammation in basophils from subjects with allergic asthma. *Respir Res* 2016; 17: 5.
- 15) CHEN Y, WONG GW, LI J. Environmental exposure and genetic predisposition as risk factors for asthma in China. *Allergy Asthma Immunol Res* 2016; 8: 92-100.
- 16) GHOSH R, ROSSNER P, HONKOVA K, DOSTAL M, SRAM RJ, HERTZ-PICCIOTTO I. Air pollution and childhood bronchitis: interaction with xenobiotic, immune regulatory and DNA repair genes. *Environ Int* 2016; 87: 94-100.
- 17) FU J, JIANG D, LIN G, LIU K, WANG Q. An ecological analysis of PM2.5 concentrations and lung cancer mortality rates in China. *BMJ Open* 2015; 5: e009452.
- 18) LI R, KOU X, XIE L, CHENG F, GENG H. Effects of ambient PM2.5 on pathological injury, inflammation, oxidative stress, metabolic enzyme activity, and expression of c-fos and c-jun in lungs of rats. *Environ Sci Pollut Res Int* 2015; 22: 20167-20176.
- 19) CARLSTEN C, MACNUTT MJ, ZHANG Z, SAVA F, PUI MM. Anti-oxidant N-acetylcysteine diminishes diesel exhaust-induced increased airway responsiveness in person with airway hyper-reactivity. *Toxicol Sci* 2014; 139: 479-487.
- 20) NITSCHKE M, PILOTTO LS, ATTEWELL RG, SMITH BJ, PISANIELLO D, MARTIN J, RUFFIN RE, HILLER JE. A cohort study of indoor nitrogen dioxide and house dust mite exposure in asthmatic children. *J Occup Environ Med* 2006; 48: 462-469.