Subchronic to Chronic Pulmonary Effects of Low Dose of Diesel Exhaust Particles in Mice after Single Intratracheal Instillation: Pathological Changes and Metallothionien Responses

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Abstract

To study the subchronic and chronic lung effects of low dose of Diesel Exhaust Particles (DEPs), mice were intratracheally instilled with 50 µg of DEPs. Cellular and biochemical parameters in bronchoalveolar lavage fluid (BALF) and histological alteration were determined at 90, 120, 150 and 180 days after instillation. From BALF results, exposure to 50 µg of DEPs did not cause any pulmonary inflammation. However, mild to moderate pulmonary changes evidenced by multifocal DEPs laden alveolar macrophages (AMs) aggregation, type II alveolar cell proliferation, thickening of alveolar wall, interstitial fibrosis and edema were observed in treated mouse lungs. Basement (BM) damage detected by laminin immunohistochemistry and myofibroblast proliferation detected by smooth muscle actin immunoreactivity on the lungs remained in the alveolar region until chronic stage. Metallothionein expression in DEPs-treated mice occurred in lung epithelial cells and laden AMs, which correlated with lung lesions. These findings suggest that instillation of a low dose of DEPs causes mild to moderate pulmonary changes and tissue damage related to the existence of DEPs.

บทคัดย่อ

การศึกษาทางพยาธิวิทยาและการปรากฏของโปรตีนเมทัลโลไธโอนีนในระบบทางเดินหายใจของหนูเมาส์ที่สัมผัสอนุภาคฝุ่นดีเซลจากท่อไอเสียในระยะกึ่งเรื้อรังและเรื้อรัง

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วัตถุประสงค์ของการศึกษาครั้งนี้ คือ เพื่อศึกษาผลกระทบต่อระบบทางเดินหายใจในหนูเมาส์ที่สัมผัสอนุภาคฝุ่นดีเซลจากท่อไอเสีย (Diesel Exhaust Particles; DEPs) ระดับต่ำในระยะกึ่งเรื้อรังและเรื้อรัง หนูได้รับการสอดท่อผ่านทางหลอดลมแล้วหยดอนุภาคฝุ่นดีเซลจากท่อไอเสียขนาด 50 ไมโครกรัม จากนั้นในวันที่ 90 120 150 และ 180 ทำการการุณยฆาตและเก็บตัวอย่างน้ำล้างปอด จากผลการวิเคราะห์ตัวอย่างน้ำล้างปอดพบว่า หนูที่ได้รับ DEPs ขนาด 50 ไมโครกรัมต่อตัวไม่มีการอักเสบของปอด แต่ในการศึกษาทางพยาธิวิทยาพบว่า การได้รับสารดังกล่าวส่งผลกระทบต่อปอด โดย พบการสะสมของเซลล์แมคโครฟาจที่เก็บกิน DEPs การเพิ่มจำนวนของเซลล์เยื่อบุถุงลมฝอยชนิดที่ 2 การหนาตัวของผนังถุงลมฝอย การเกิดพังผืดของผนังถุงลมฝอย และการบวมน้ำของปอด นอกจากนี้ยังพบความเสียหายของเยื่อยึดฐานโดยการย้อมด้วยอิมมูนโนฮิสโตเคมีของลามินีนและการเพิ่มขึ้นของเซลล์ไมโอไฟโบรบลาสต์จากการย้อมด้วยอิมมูนโนฮิสโตเคมีของ smooth muscle actin จากการศึกษาการแสดงออกของโปรตีนเมทัลโลไธโอนีน พบการติดสีบริเวณเซลล์เยื่อบุของปอดและเซลล์แมคโครฟาจที่เก็บกินอนุภาคในบริเวณที่พบรอยโรคของปอด จากผลการทดลองทั้งหมดสรุปได้ว่า การสัมผัสอนุภาคฝุ่นดีเซลจากท่อไอเสียในระดับต่ำส่งผลให้ปอดของหนูเสียหายในระยะกึ่งเรื้อรังและเรื้อรัง

คำสำคัญ: ระยะเรื้อรัง อนุภาคฝุ่นดีเซลจากท่อไอเสีย การหยดสารผ่านหลอดลม ระยะกึ่งเรื้อรัง ผลกระทบต่อระบบทางเดินหายใจ

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Introduction

Diesel exhaust particles (DEPs), carbon-based particles that contain a variety of polyaromatic hydrocarbons (PAHs) and traces of heavy metals, are one of the major sources of atmospheric PM 2.5 in urban areas (Schuetzle, 1983; Schuetzle and Lewtas, 1986; Ghio et al., 2000). DEPs can cause inflammation of respiratory system and thrombosis of artery and vein (Mauderly et al., 1987; McClellan, 1987; Ichinose et al., 1995; Nemmar et al., 2003). Epidemiologic studies of occupationally DEPs exposed workers showed prevalence of respiratory symptoms and impaired pulmonary function consistent with obstructive airway disease. Moreover, exposure to DEPs might increase risk of cardiovascular disease (Saito et al., 2002). Chronic exposures of DEPs to laboratory animals can cause lung injury, altered pulmonary function and increased DEPs retention in the lung. It is also apparent that long term exposures to DEPs also have the potential to increase susceptibility to respiratory tract infection, neurological or behavioral changes, an increase in banded neutrophils, and morphological alterations in the liver (Sagai et al., 1996; Nemmar et al., 2010). After prolonged exposures to DEPs , impairment of pulmonary function including lung mechanical properties, diffusing capacity, lung volumes, and ventilatory performance has been reported in rats, hamsters, cats, and monkeys (Heinrich et al., 1986; U.S. EPA, 2002). Various factors associated with the disparity of the pulmonary functions involve dose of particles, site of deposition, retention in the lung and host's clearance or repair mechanism (Yoshizaki et al., 2010).

Standard reference materials (SRMs) of DEPs (SRM 2975) that were collected from an industrial diesel-powered forklift could induce acute lung inflammatory responses on aspiration or instillation (Singh et al., 2004; Kaewamatawong et al., 2009). To our knowledge, subchronic and chronic effects of SRM 2975 are still not clarified.

Metallothionein (MT) is a low molecular weight and cystein-rich protein that can regulate essential metals such as Zn and plays an important role in detoxification of non-essential metal ions such as Cd, Pb and Hg (Chan, 1995; Cheung et al., 2004; Lau et al., 2001; Shimada et al., 2005; Wu et al., 2008; Gao et al., 2009). The protective role of MT to DEPs is still largely unknown. Park and Moon (2004) reported that DEPs could induce the expression of MT gene in human lung epithelial cell line. From our knowledge,

there is no report of MT expression in *in vivo* study caused by exposure to DEPs.

The aim of this study was to clarify biological and pathological changes of intratracheally instilled low dose of DEPs on the lungs of mice during the subchronic and chronic stages using BAL techniques, histopathology and immunohistoche- mistry. Moreover, the protective role of metallothionein and the clearance time point during subchronic and chronic phase were also elucidated.

Materials and Methods

Experimental animal: Male ICR mice, weighing 35-38 g and 7-8 weeks of age, were purchased from National Laboratory Animal Centre, Mahidol University. The mice were housed in an animal facility under 12:12 hour light/dark cycle, temperature of 24 ± 1^0C, relative humidity of $55\pm10\%$ and negative atmospheric pressure. They were provided with mouse chow and filtered tap water ad libitum throughout the experiment. All protocols of animal experiments were certified by the ethics committee of Chulalongkorn University Animal Care and Use Committee (CU-ACUC).

Particles: Standard reference materials (SRMs) of DEPs (SRM 2975) that were collected from an industrial diesel-powered forklift had been certified by the National Institute of Standards Technology (NIST, Gaithersburg, MD, USA). According to NIST analyses, SRM 2975 samples consisted of polycyclic aromatic hydrocarbons (PAHs), 1- nitropyrene compounds and some traces of minerals. The SRM 2975 particles were fine polygonal shape with diameters approximately < 10 μm. The specific surface area was 91 m^2/g.

Experimental design: Eighty-four Male ICR mice were divided randomly into 8 groups of 9-12 animals each. In treatment groups, mice were single intratracheally instilled with 50 μl aqueous suspensions of 50 μg of SRM 2975 suspended in 0.01 M phosphate-buffered saline (PBS). The control groups of mice were instilled with 50 μl of 0.01 M PBS. At 90, 120, 150 and 180 days after instillation, the animals in each group were sacrificed for BALF collection (7-9 mice) and histopathological evaluations (2-3 mice).

Bronchoalveolar lavage analysis: Bronchoalveolar lavage fluid was collected from the lungs according to our previous study (Kaewamatawong et al., 2006). Briefly, mouse was scarified and the trachea was cannulated. Three injections of 1 ml of NSS were lavaged and harvested from the lung. The recovery rate of BALF was calculated. The average fluid recovery was greater than 90% of the amount instilled. The collected fluid was centrifuged at 3,000 rpm for 10 minutes at 4^0C and the supernatants were stored at -80^0C until analysis. The cell pellets were quantified by hemocytometric counting for the total number of cells and cell viability. Cell viability was assessed by exclusion of tryphan blue dye.

Cell differentiation was evaluated on cytologic preparations from BALF pooled from each mouse by Diff-Quik stain (Sysmex®, Kobe, Japan). A total of 200 cells were determined under light microscopy.

Histopathology: Various organs such as lung, hilar lymph node, heart, liver and kidney were preserved in 10% buffered neutral formalin after gross evaluation. The routine histological processes were performed.

The 3 μm thick of lung's sections and 4-5 μm thick of other organ's sections were cut and stained with hematoxylin and eosin (H&E). The histopathological changes in lung and lymph node were determined for lesion severity as follows: 0 = no significant finding; 1 = very slight degree; 2 = slight degree; 3 = moderate degree; 4 = severe degree. Blind evaluation method was used to evaluate by two veterinary pathologists in a blind manner.

Immunohistochemistry protocol:

Laminin: Laminin immunohistochemistry was performed to detect basement membrane in the lung, using monoclonal rabbit anti laminin antibody (Dako®, Glostrup, Denmark) as the primary antibody with EnVisionTM detection system (Dako®, Glostrup, Denmark). After deparaffinization, the sections were treated with proteinase K for 30 min at 37^0C citrate and incubated with 3% H_2O_2 to quench endogenous peroxidase for 15 min at room temperature. The sections were blocked with 10% normal goat serum for 5 min in a 250 watt microwave oven 250 watt to inhibit nonspecific reactions. After the primary antibodies of monoclonal mouse, polyclonal rabbit aniti-laminin monoclonal antibody diluted at 1:200 (DAKO, Glostrup, Denmark) were applied to the sections. The sections were kept at 4^0C over night. The biotinylated anti-mouse IgG antibody and EnVision polymer (Dako REAL™ EnVision™ detection system, Dako®, Denmark) reacted to sections as a secondary antibody in a 200 watt microwave oven for 7 min. The brown staining with the substrate 3,3′- diaminobenzidine tetrahydrochloride (DAB) was determined as the positive result and the sections were counterstained with hematoxylin.

Smooth muscle actin: Smooth muscle actin expressing myofibroblasts in animal lungs were observed using monoclonal mouse anti-smooth muscle actin. After deparaffinization and rehydration, sections were pretreated with citrate buffer solution (pH 6.0) and microwaved. The sections was quenched with 3% H_2O_2 at room temperature for 30 min and blocked with 10% normal goat serum for 5 min with microwave treatment. 1:50 dilution of monoclonal anti-smooth muscle actin (Dako, Glostrup, Denmark) was incubated on the tissue over night at 4^0C. After primary antibody reaction, the peroxidase-labeled polymer conjugated to secondary antimouse antibodies (EnVision® kit/HRP (DAB), Dako, Glostrup, Denmark) were added to the sections for 30 min at room temperature. The brown positive reaction was visualized by addition of substrate 3,3′- diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained with hematoxylin.

Metallothionein: Tissue sections from lung were immunostained to detect metallothionein protein,

using monoclonal mouse metallothionein antibody (MT1; E9, Dako®, Glostrup, Denmark) with EnVisionTM detection system (Dako®, Glostrup, Denmark). After deparaffinization, the sections were placed in citrate buffer solution pH 6.0 for 20 min by microwave heat at 700 watt for 5 min for antigen retrieval process. For inhibit nonspecific reactions, the sections were incubated with 3% H_2O_2 in methanol for 30 min at room temperature and then with 1% BSA for 30 min at 37^0C. Thereafter, the sections were reacted over night at 4^0C with mouse anti-horse metallothionein monoclonal antibody diluted at 1:50. For the secondary antibody, the biotinylated anti-mouse IgG antibody and EnVision polymer (Dako REALTM EnVision™ detection system, Dako®, Denmark) reacted to sections at room temperature for 45 min. The positive brown reactions were developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB). Hematoxylin was used for counter staining. Tissue that was used as positive control for metallothionein protein in this study was human ductal carcinoma (Shimada et al., 2005).

Statistical analysis: Data of BALF analysis were presented as means±standard error (SE). Statistical analysis was determined by Student's t-test. Significant differences between means were regarded as p value of less than 0.05 ($p < 0.05$).

Results

Bronchoalveolar lavage fluid analysis

Following instillation of 50 μg DEPs, the numbers of total cells (Fig 1), viable cells (Fig 2), macrophages (Fig 3) and lymphocytes (Fig 4) in BALF at 90, 120, 150 and 180 days post-exposure showed no significant difference in any time points.

Histopathology

The severity of histopathological changes in lung and lymph node were summarized in Table 1. No significant changes were found in the lungs of control mice in all observation times (Fig 5). By contrast, at 90, 120, 150 and 180 days after instillation, a number of nodular aggregates of particle-laden AMs were observed in some alveolar regions adjacent to the bronchioles and blood vessels (Fig 6). Alveolar septal walls adjacent to the aggregated nodules thickened due to type II pneumocyte regenerative hyperplasia, interstitial accumulation of macrophages and fibrosis. Increase in neutrophils in capillary vessels in alveoli was also occasionally seen in some areas of treated lungs. At 180 days after instillation, changes in the lungs were restricted to the appearance of multifocal small aggregated nodules. Some small black dots of DEPs were found in cytoplasm of alveolar peumocyte cells even type I or type II cells at all time points (Fig 7). The accumulation of particle-laden macrophages and hyperplastic histiocytes in subcapsular and medullary sinus of hilar lymph nodes of treated animals was observed at all observation times. The severity of histopathological changes in lung and lymph node was summarized in Table 1.
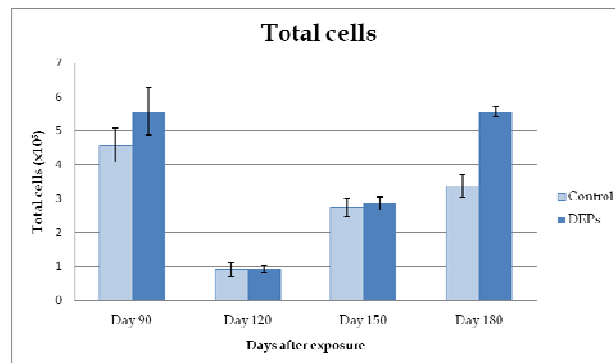

Figure 1 Chronic effect of DEPs exposure on BAL total cell counts ($x10^5$). *All data are expressed as means±SE and were compared with the ANOVA (n = 5). Differences with $p < 0.05$ are considered statistically significant.
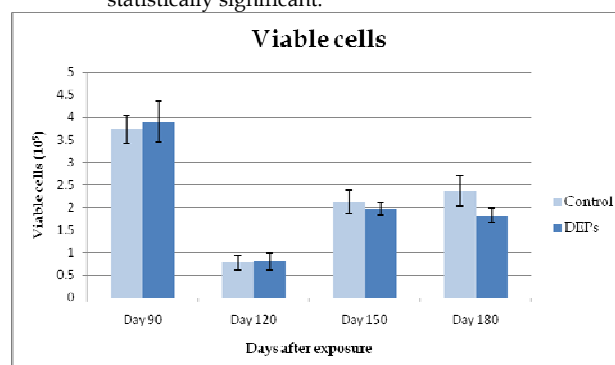

Figure 2 Chronic effect of DEPs exposure on BAL viable cell counts ($x10^5$). *All data are expressed as means±SE and were compared with the ANOVA (n = 5). Differences with $p < 0.05$ are considered statistically significant.
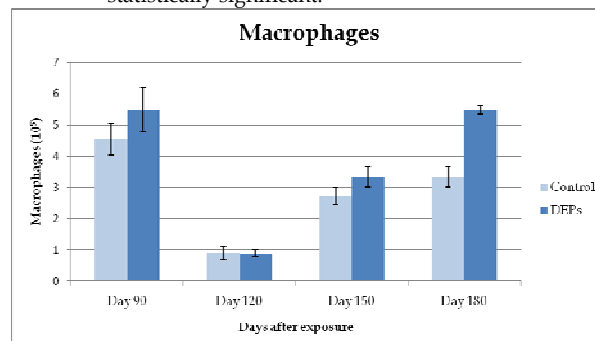

Figure 3 Chronic effect of DEPs exposure on BAL macrophage counts ($x10^5$). * All data are expressed as means±SE and were compared with the ANOVA (n = 6). Differences with $p < 0.05$ are considered statistically significant.

Figure 4 Chronic effect of DEPs exposure on BAL lymphocyte counts ($x10^5$). *All data are expressed as means±SE and were compared with the ANOVA (n = 6). Differences with $p < 0.05$ are considered statistically significant.
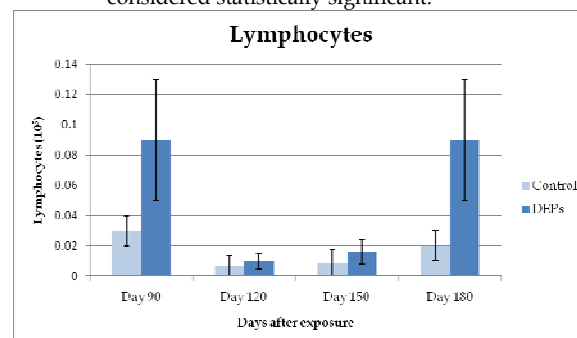
Laminin

In the control animals, basement membranes of alveolar septa, bronchus, bronchiole, blood vessel and bronchial gland showed thin string-like lines of brown positive stain of laminin (Fig 8A). Extensive patchy areas of non-stain or weak positive stain of alveolar basement membranes were seen in site of the aggregated nodules. Brown faint discontinuous lines of BM were also observed in the areas close to the nodules (Fig 8B).

Smooth muscle actin

In the control groups, the positive brown staining distributed around major airways and blood vessels. It also stained very weakly at the alveolar septa (Fig 9A). In contrast, very strong smooth muscle actin immunoreactivity was seen in thickened alveolar walls of the lungs from the treated animals. (Fig 9B). The positive reaction surrounding the airways and blood vessels were did not remarkably change compared to the controls.
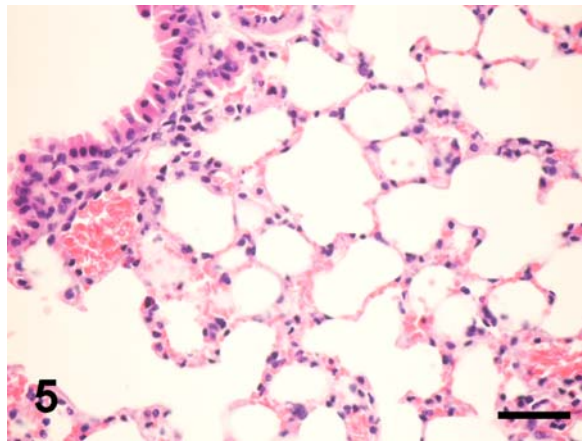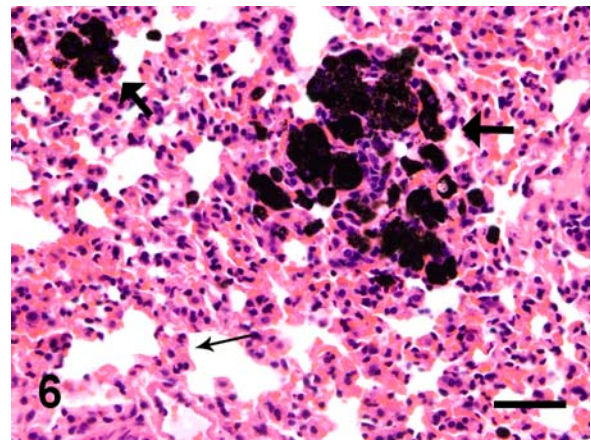

Figure 6 Lung section from DEPs treated groups at 120 days post-exposure reveals multifocal aggregation of DEPs laden AMs (large arrows) and thickening of alveolar septal walls (small arrow), H&E stain, Bar = 600 µm.
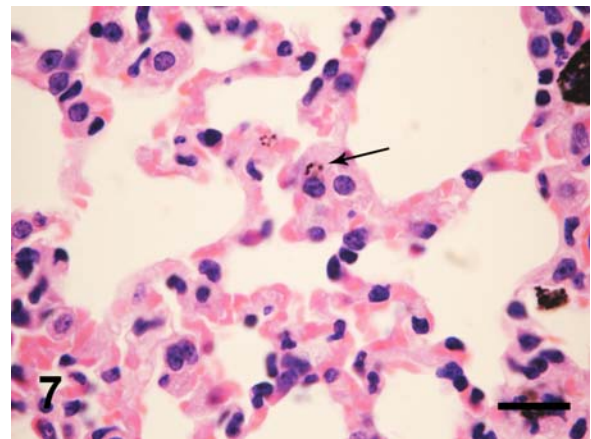

Figure 5 Lung section from control group shows normal structure of bronchioe and alveoli, (Bar = 500 µm).

Figure 7 Lung section from DEPs treated groups at 150 days post-exposure shows DEPs particles (arrow) in cytoplasm of alveolar epithelial type II cell, H&E stain, Bar = 700 µm.

Table 1 Histopathological assessment of the lesion severity in lungs and lymph nodes of control and 50 µg DEPs-treated mice

Lesions	90 days		120 days		150 days		180 days	
	Control	DEPs	Control	DEPs	Control	DEPs	Control	DEPs
Alveolar lesions								
- congestion	2-3	3	2-3	3	2-3	2-3	1	2
- increasing number of neutrophils in alveolar wall	1	2	1	1-2	0-1	1	1	1
- increasing cells in air space								
- active AMs	0	0	0	0	1	1	0	0
- laden AMs	0	0	0	0	0	1	0	1
- laden AMs infiltrate through alveolar wall	0	2	0	1	0	1-2	0	1
- granulomatous nodules								
- number of nodules	0	1	0	1	0	1-2	0	1
- type II pneumocyte swelling	1-2	2-3	0	2	1-2	2	2	2
- laden type II pneumocytes	0	1-2	0	1	0	1	0	1
- regenerative hyperplasia of type II pneumocytes	1	1-2	0	1	1	1	1	1
Bronchial & bronchiolar lesions								
- laden AMs in lumen or attach on epithelial cells	0	1	0	1	0	1	0	0
Perivascular lesions								
- laden AMs accumulate around vessels	0	1	0	0	0	1	0	0
BALT lesions								
- hyperplasia	0	0	1	1	1	1-2	1	1-2
Lymph node's lesions								
- laden AMs infiltration	0	2	0	2-3	0	2	0	1
- sinus histiocytosis	1	1	0	2	0	0	0	0

0: no significant finding, 1: very slight, 2: slight, 3: moderate, 4: severe degree
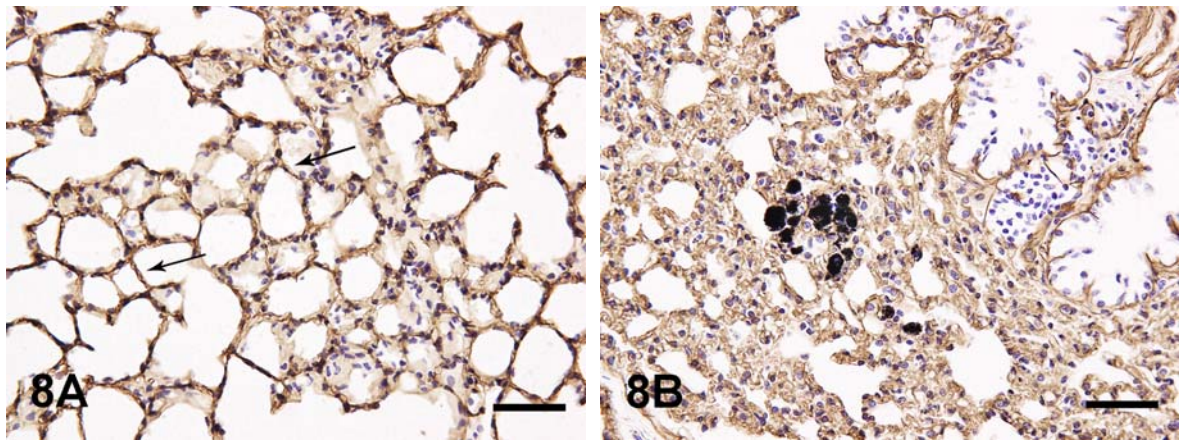AMs: alveolar macrophages, CNT: connective tissue

Figure 8 Laminin immunohistochemistry in lungs of control and DEPs-treated mice sacrificed at 120 days post-exposure. (A) Brown thin string-like positive staining was localized along alveolar basement membranes (arrows) in control animals. (B) Weak and discontinuous positive patterns of alveolar basement membrane were observed inside and adjacent to aggregation of DEPs laden AMs in DEPs-treated mice, Bar = 500 μm.
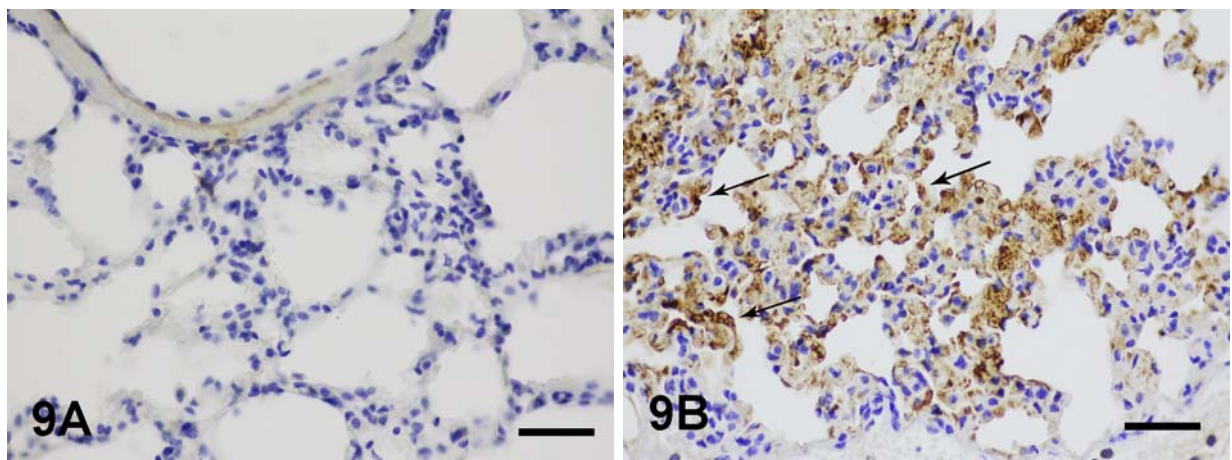

Figure 9 Immunohistochemical staining for smooth-muscle-specific actin. A: control group, B: DEPs exposure group (Bar = 500 μm). Immunohistochemical analysis revealed larger areas of myofibroblasts as determined by expression of a-SMA (arrows) in lungs of mice treated with low doses of DEPs than in lungs of control mice.
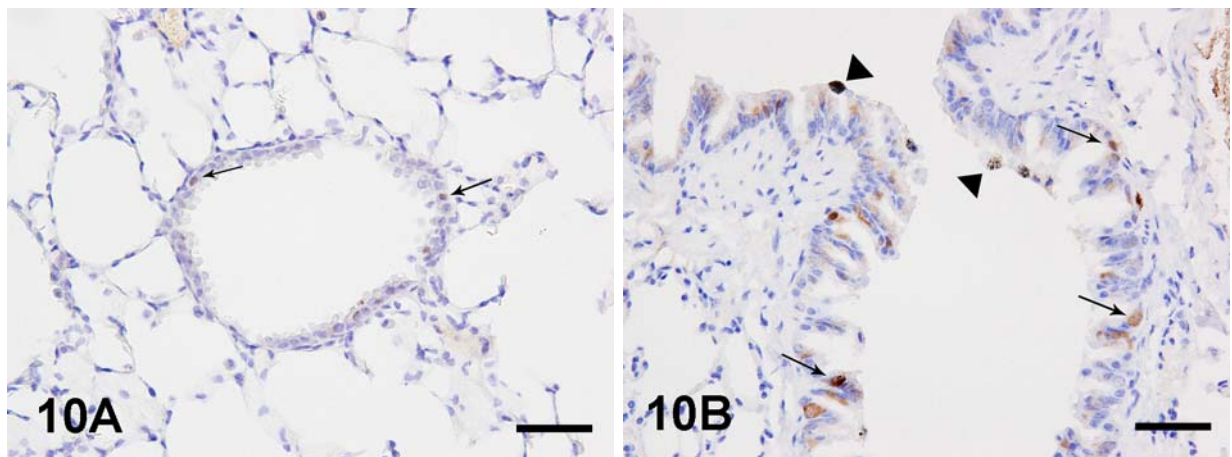

Figure 10 Immunohistochemical localization of MT in lungs of control and DEPs-treated mice killed at 180 days post-exposure. (A) Brown positive staining was occasionally observed in cytoplasm of bronchiolar epithelium in control animals (arrow), Bar = 500 μm. (B) In DEPs -treated mice, intense brown immunostaining appeared in the cytoplasm of bronchiolar epithelial cells (arrows) and DEPs laden AMs (arrowheads), Bar = 600 μm.

Metallothionin

In the control lungs, the immunohistochemical staining of MT was barely detectable in the cytoplasm of bronchi and bronchiolar epithelium (Fig 10A). By contrast, in the DEPs-treated mice at 90 to 180 days post exposure, positive staining for MT appeared in a large number of cells associated with aggregated nodules and areas that were close to laden AMs. MT expressed mainly in the cytoplasm of bronchiolar epithelial cells, alveolar epithelial cells and laden AMs (Fig 10B).

Discussion

In our previous study, we described acute pulmonary pathological effects caused by single intratracheal exposure to low doses of DEPs in mice. The results showed that instillation of low dose of DEPs (50 µg) induced mild to moderate pulmonary inflammation and injury correlated with the particle accumulation. At the subacute stage, almost all of the pulmonary lesions gradually recovered to normal appearances. However, DEPs granulomas and alveolar basement membrane damages were still seen in some areas of treated mouse lungs. The effects of low dose of DEPs (SRM 2975) on bronchoalveolar lavage indices suggested that DEPs did not induce any pulmonary inflammation at subchronic and chronic stages. Concomitant histopathological findings generally correlate with BALF data. However, some pulmonary changes including multifocal DEPs aggregated nodules, alveolar type II pneumocyte hyperplasia, thickened alveolar septa and interstitial fibrosis were still observed in treated animals. The distribution of DEPs was also found in cytoplasm of alveolar AMs and alveolar epithelial cells at all time points. In hilar lymph nodes, the accumulation of particle-laden macrophages was the major evidence. These findings were quite similar to other chronic experiment that studied the exposure to other kinds of DEPs (U.S. EPA, 2002).

To study the alveolar basement membrane damages, laminin immunohistochemistry was performed to the lungs. Non-stain or weak positive stain of laminin protein expression was observed at the areas of DEPs aggregated nodules and discontinuous lines of alveolar BM were seen at the site adjacent to the nodules. These finding indicated that low dose of DEPs could induce alveolar BM damages at subchronic and chronic stages. In our previous study of subscute effects of DEPs, we reported the ultrastructural changes of dissociation of alveolar basement membranes that were associated with particle accumulation (Kaewamatawong et al., 2009). We believed that DEPs could cause long term effect to the alveolar BM as evidences that exit from acute to chronic experiments.

Myofibroblasts have been considered to play an important role in tissue injuries, remodeling processes and fibrocontractive diseases (Zhang et al., 1994). The morphology of myofibroblasts is intermediate between fibroblasts and smooth muscle cells with highly synthetic activity (Hinz and Gabbiani, 2010). In the development of tissue fibrosis, the proliferation of myofibroblasts continues from the normal tissue repair process and induces excessive production and accumulation of extracellular matrix. Alpha-smooth muscle actin (αSMA) has been reported as a marker for myofibroblastic cells (Ohta et al., 1995). The persistence of αSMA expressing myofibroblasts involves in hypertrophic scars and in fibrotic lesions of many organs, including lung (Sime and O'Reilly, 2001). In our study, we demonstrated the intense positive activity of smooth muscle actin in some areas of the thickening of alveolar septal walls of mice exposed to DEPs. These results implied that low dose of DEPs could cause the proliferation of myofibroblasts and might be contribute to pulmonary interstitial fibrosis.

Metallothionein (MT) has been considered to play a crucial role in regulation and detoxification of heavy metals. Moreover, MT can also react with oxidative stress and protects against free radical–induced cell damage. *In vitro* study of MT expression induced by DEPs was determined in human lung epithelial cell line (Park and Moon, 2004). The MT expression of DEPs in *in vivo* experiment was not reported previously. In our study, MT expression was detected in airway epithelial cells that were associated with DEPs aggregated nodules and areas that were close to laden AMs. We, therefore, concluded that MT might have a protective role to DEPs at subchronic and chronic stage. The heavy metals such as Zn, Cu and Fe in DEPs played an important role to induce the expression and the protection of MT in our study.

Clearance of particles from the alveolar region after exposure to DEPs at subchronic and chronic stages reduced in this study. The concomitant multifocal DEP aggregated nodules characterized by the aggregations of particle-laden AMs were observed particularly in the peribronchiolar and alveolar regions, as well as in the hilar lymph nodes. The factors that cause the impairment of DEPs clearance depend on animal species, exposure concentration, uptake rate, rate of pulmonary deposition, pulmonary clearance rate, population of AMs per lung unit, rate of recruitment of AMs and leukocytes, function of AMs and efficiencies to remove particles via the mucociliary and lymphatic clearance system (U.S. EPA, 2002). In our results, the clearance time of DEPs was more than 180 days post-exposure due to the fact that the evidence of the agglomeration of particle-laden AMs remained in the alveolar region. However, longer-term chronic toxicity study should be performed in order to gain a more complete understanding of clearance time.

In summary, this study demonstrated the pulmonary biological and pathological responses after intratracheal instillation of low dose of DEPs in mice during the subchronic and chronic stages. Low dose of DEPs did not produce pulmonary inflammation by BALF data. However, histopathology of pulmonary changes including DEPs aggregated nodules, agglomeration of particle-laden AMs, BM damage, the proliferation of myofibroblasts and interstitial fibrosis on the lungs remained in the alveolar region until chronic stage. Furthermore, our current study found that DEPs could induce the expression of MT that might be one of the protective mechanisms of lung against DEPs.

Acknowledgements

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