

Acute and Subacute Pulmonary Effects of Diesel Exhaust Particles in Mice: Pathological Changes and Translocation Pathways to the Circulation

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Abstract

To study the acute and subacute pulmonary effects of diesel exhaust particles (DEPs), mice were intratracheally instilled with 25, 50 or 100 μ g of DEPs for dose response experiments. Histological alterations were determined at 3 days post-exposure. 50 or 100 μ g of DEPs produced mild to moderate pulmonary inflammation and tissue injury characterized by infiltration of neutrophils and active alveolar macrophages (AMs), focal alveolitis and particle-laden AMs accumulation. Ultrastructural studies of treated animals showed the dissociation of basement membranes and erosion of type I alveolar epithelial cells. To investigate the time response, mice were instilled with 50 μ g of DEPs and sacrificed at intervals from 1 to 30 days post-exposure. DEPs induced pulmonary inflammation and injury at acute period; however, these changes gradually regressed during the experiment. These results suggest that instillation of small doses of DEPs causes transient acute mild to moderate lung inflammation and tissue damage. The evidences of the DEPs distribution in lung tissues were also elucidated throughout the observation time. The main possible translocation pathway of DEPs from the lung to the circulation in this study could be cell mediated active transportation and direct penetration through the area of alveolar interstitial damages.

Keywords : acute, diesel exhaust particles, intratracheal instillation, pulmonary effects, subacute

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บทคัดย่อ

การศึกษาทางพยาธิวิทยาและการแพร่ผ่านของอนุภาคเข้าสู่กระแสเลือดในระบบทางเดินหายใจของหนูเม้าส์ที่มีผลจากการสัมผัสอนุภาคฝุ่นดีเซลจากห้องเผาไหม้ในระยะเฉียบพลัน และกึ่งเฉียบพลัน

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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาพยาธิวิทยาของระบบทางเดินหายใจในหนูเม้าส์ที่มีผลจากการสัมผัสอนุภาคฝุ่นดีเซลจากห้องเผาไหม้ในระยะเฉียบพลันและกึ่งเฉียบพลัน รวมทั้งได้ทำการศึกษาการแพร่ผ่านของอนุภาคดังกล่าวจากระบบทางเดินหายใจเข้าสู่ระบบไฟล์เวียนเลือด โดยได้แบ่งการทดลองออกเป็น 2 ส่วน ส่วนแรกเป็นการศึกษาการตอบสนองต่อบริมาณที่สัมผัสโดยการสอดท่อผ่านทางหลอดลมแล้วหยดอนุภาคฝุ่นดีเซลจากห้องเผาไหม้ (Diesel Exhaust Particles (DEPs) เข้าสู่ปอดหนู จัดแบ่งกลุ่มทดลองตามปริมาณของอนุภาค (25, 50 และ 100 ไมโครกรัมต่อตัว) จากนั้นทำการผ่าตัดเพื่อศึกษาทางพยาธิวิทยาต่อไปพบว่าในหนูที่ได้รับ DEPs ขนาด 50 และ 100 ไมโครกรัมต่อตัว ทำให้เกิดการอักเสบและความเสียหายของปอดในระดับปานกลาง โดยจะพบการสะสมของเซลล์นิวโตรอฟิล และเซลล์เม็ดโคโรฟาร์เจอร์ บริเวณที่มีอนุภาค DEPs สะสมอยู่ และพบการกระชายหัวไวของอนุภาคในเซลล์ของปอด ความรุนแรงของรอยโรคขึ้นกับปริมาณของอนุภาคที่ได้รับเข้า จากการศึกษาทางกล้องจุลทรรศน์ วิธีเล็กตระหนัลบพบความเสียหายของเยื่อบาสเมนท์และการลอกหดของเซลล์เยื่อบุถุงลมฟอยชนิดที่ 1 การทดลองที่ 2 เป็นการศึกษาการตอบสนองในระยะเฉียบพลันและกึ่งเฉียบพลันของอนุภาค DEPs โดยการหยดอนุภาค DEPs ขนาด 50 ไมโครกรัมต่อตัว แล้วทำการศึกษาทางพยาธิวิทยาตั้งแต่วันที่ 1 จนถึงวันที่ 30 ของการทดลอง ในระยะเฉียบพลัน (1-3 วัน) หนูในกลุ่มทดลองพบการอักเสบและความเสียหายของปอดในระดับปานกลาง จากนั้นในระยะเวลาอีกมาจะพบรอยโรคต่างๆมีความรุนแรงลดลง จนกระทั่งถึงวันที่ 30 ของการทดลองยังพบรอยโรคต่างๆอยู่เพียงเล็กน้อย จากการศึกษาทางกล้องจุลทรรศน์วิธีเล็กตระหนัลพบว่าอนุภาคในปอด พนอนุภาคกระชายไว้ปิดตามโครงสร้างต่างๆของปอด รวมทั้งถูกเก็บกินจากเซลล์เม็ดโคโรฟาร์เจอร์ จากการทดลองนี้พบว่าอนุภาค DEPs ปริมาณน้อย สามารถกระตุ้นทำให้เกิดการอักเสบในปอดได้ และจากการศึกษาการแพร่กระจายของอนุภาคพบว่ามีแนวโน้มที่อนุภาค DEPs จะสามารถผ่านเข้าสู่ระบบไฟล์เวียนเลือดได้ โดยการแพร่ผ่านทางเซลล์เยื่อบุถุงลมฟอยและจากการถูกเก็บกินโดยเซลล์เก็บกิน รวมทั้งอาจจะแพร่ผ่านโดยตรงที่บริเวณที่เกิดความเสียหายของเซลล์เยื่อบุถุงลมฟอยซึ่งเป็นผลมาจากการสัมผัสกับอนุภาคฝุ่นดีเซลจากห้องเผาไหม้

คำสำคัญ : ระยะเฉียบพลัน อนุภาคฝุ่นดีเซลจากห้องเผาไหม้ การหยดสารผ่านหลอดลม ผลกระทบต่อระบบทางเดินหายใจ และระยะกึ่งเฉียบพลัน

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Introduction

Exposure to urban pollution, especially by particles, has been associated with a number of adverse health effects, including cardiopulmonary morbidity and mortality and lung cancer mortality (Dockery et al., 1993; Peters et al., 1999; Samet et al., 2000). In urban areas, diesel exhaust particles (DEPs) derived from diesel engine-powered automobiles are a major source of atmospheric PM2.5. DEPs are carbon-based particles that contain a variety of organic compounds such as polyaromatic hydrocarbons (PAHs), nitro-PAHs, quinines,

heterocyclics, aldehydes, pyrenes and traces of heavy metals (Schuetzle, 1983; Schuetzle and Lewtas, 1986; Ghio et al., 2000). Previous studies showed that DEPs cause pulmonary inflammation and aggravate arterial and venous thrombosis (Mauderly et al., 1987; McClellan, 1987; Ichinose et al., 1995; Nemmar et al., 2003). In human, a short-term exposure to DEPs induces inflammatory responses in the airways and the peripheral blood (Salvi et al., 1999; Salvi et al., 2000). In vitro, DEPs are taken up by airway epithelial cells and stimulate the release of proinflammatory cytokines (Boland et al., 1999).

Comparison among health effect studies of DEPs can be complicated by variability in the chemical composition of the particles, which is influenced by the age and type of engine, fuel composition, load characteristics, lube oil components, presence and efficiency of emission control devices and sampling procedures (Schuetzle and Lewtas, 1986). Consequently, the biologic activities of sample generated and collected under different conditions are likely to be different.

Standard reference materials (SRMs) of DEPs (SRM 2975) that collected from an industrial diesel-powered forklift have been certified by the National Institute of Standards Technology (NIST, Gaithersburg, MD, USA) for use in evaluating analytical methods for the determination of selected PAHs in diesel particulate matter and similar matrices (Claxton et al., 1992). All of the chemical constituents provided in SRM 2975 are naturally present in particulate material. SRM 2975 was developed in part for mutagenicity assays (Hughes et al., 1997; DeMarini et al., 2004) and showed acute lung inflammatory responses on aspiration (Singh et al., 2004). However serial pathological changes in lung at acute and subacute stages are still not clarified.

Epidemiological studies have shown that the increase in mortality linked to particulate matter $< 10 \mu\text{m}$ in diameter is attributable to cardiovascular functional disturbances and diseases (Pope et al., 1999). Although these clinical and epidemiological observations are strong and consistent, the underlying mechanisms responsible for the cardiovascular toxicity of particulate matter are still largely unknown. Several mechanisms responsible for the cardiovascular effects of particulate matters have been hypothesized. One of the hypotheses to explain these effects is that the particles cause inflammatory responses in the lung leading to release of mediators, which may influence the heart, coagulation, or other cardiovascular endpoints (Seaton et al., 1995). Another alternative hypothesis, which has not been much investigated so far, is that the particles translocate from the lungs into systemic circulation and thus influence hemostasis or cardiovascular integrity more directly. Studies suggest that PM2.5 have an important role in triggering biological responses and remain airborne for long periods of time. These particles can penetrate deeply into the respiratory tract and carry large amount of toxic compounds. A

number of morphological studies have been demonstrated several translocation pathways of particulate particles (Takenaka et al., 2001; Shimada et al., 2006). However, there is no any morphological report on the translocation of DEPs from the lung to systemic circulation.

The purpose of this study is to describe acute and subacute pulmonary pathological effects caused by intratracheal exposure to DEPs (SRM 2975) and elucidate the possible translocation pathways from the lung into the systemic circulation using light and electron microscopy.

Materials and Methods

Particles: Standard reference materials (SRMs) of DEPs (SRM 2975) that collected from an industrial diesel-powered forklift have been certified by the National Institute of Standards Technology (NIST, Gaithersburg, MD, USA). According to NIST analyses, SRM 2975 samples consist of polycyclic aromatic hydrocarbons (PAHs) and 1-nitropyrene compounds. The scanning electron microscopic images of SRM 2975 showed fine polygonal particles with diameters approximately $< 10 \mu\text{m}$. The specific surface area, which measured by Brunauer, Emmett and Teller (BET), was $91 \text{ m}^2/\text{g}$.

Experimental design

Dose response: Dose response: To find the appropriate dose for determination of DEPs lung toxicity, the doses of 25, 50 or 100 μg of SRM 2975 were used. Forty-eight male ICR mice were randomly separated into four control and twelve exposure groups of 3 animals each. Twelve exposure groups were single intratracheally instilled with 50 μl aqueous suspensions of 25, 50 or 100 μg of SRM 2975 suspended in 0.01 M phosphate-buffered saline (PBS). The four groups of control were instilled with 50 μl of 0.01 M PBS. At 5 min, 6, 24, and 72 hr after instillation, the animals in each group were sacrificed and various organs such as lung, hilar lymph node, heart, liver and kidney were collected and preserved in 10% buffered neutral formalin for routine histopathological evaluations and 2.5% glutaraldehyde for electron microscopic examinations.

Time effect: Seventy mice were divided randomly into 30 groups of 2-3 animals each. The fifteen treated groups 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 rest of fifteen control groups of mice were instilled with 50 μ l of 0.01 M PBS. Animals in each group were sacrificed at 0, 5, 10, 30 min, 1, 2, 6, 12, 24, 48, 72 hr, 5, 7, 15, 30 days post-exposure, respectively. The tissue samples were collected as described above.

Ultrastructural studies: Using transmission electron microscope, lung tissue samples were post-fixed in 1% osmium tetroxide, dehydrated with serial alcohol and embedded in epoxy resin. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a JEM-100CX electron microscope (JEOL, Tokyo, Japan).

Results

Dose responses of SRM 2975

Clinical and gross findings: In control and all of treated animals, there were no exposure-related clinical signs in any observation time. Grossly, instillation of 50 and 100 μ g of DEPs caused mild congestion and edema in the lungs compared to 25 μ g treated and control groups.

Histopathology: No significant lesions were observed from the lungs of control animals at all observed times. However, accumulation of free aggregated particles was found in the alveoli and bronchiolar lumens of all treated groups at 5 min after instillation. Some of aggregated particles were present within alveolar macrophages, and occasionally present within alveolar epithelial cells (Figure 1A). Furthermore, increasing number of cells in alveolar wall was also observed in the lungs of all treated groups. By 6 hr after instillation, the animals instilled with 50 and 100 μ g DEPs had mild to moderate influx of neutrophils into the alveolar air spaces and into the interstitium around peribronchiolar vessels (Figure 1B). At 24 hr after the instillation, a number of nodular aggregate of neutrophils and particle-laden AMs were observed in some alveolar regions particularly in the perivasculär areas adjacent to the bronchioles. By 3 days after instillation, mild to moderate focal alveolitis characterized by accumulation of numerous active AMs, particle-laden AMs, and some neutrophils were observed

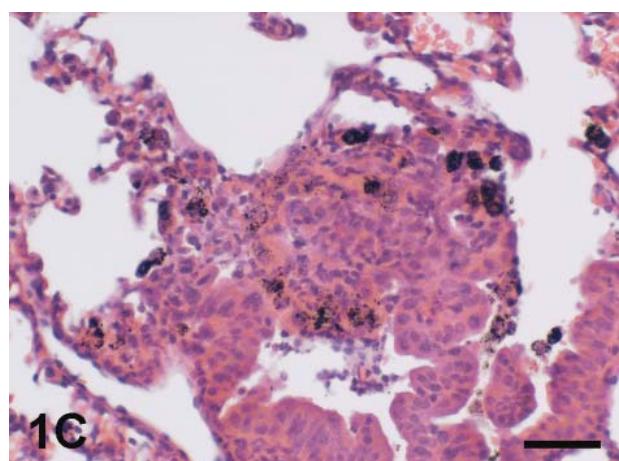
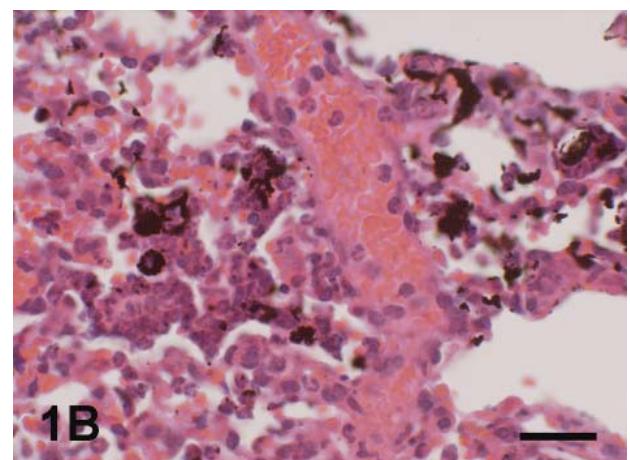
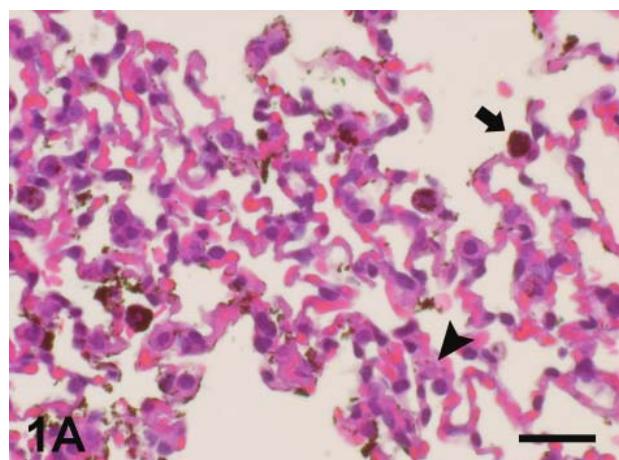


Figure 1. Lung tissues from various doses of DEPs treated groups show variety of pulmonary effects, H&E stain. (A) the distributions of DEPs in AMs (arrow) and alveolar epithelium (arrowhead); 25 μ g treated group, (bar = 500 μ m). (B) Influx of neutrophils around the DEPs accumulation; 100 μ g treated group, (bar = 40 μ m). (C) Focal alveolitis; 100 μ g treated group, (bar = 700 μ m).

at the terminal bronchiolar and alveolar duct regions (Figure 1C). The hilar lymph nodes of all treated groups were slightly enlarged with some particle-laden AMs infiltration into lymphoid parenchyma. The lesions of lungs and lymph nodes in mice treated with DEPs at the dose of 50 and 100 µg were similar in appearance but the magnitude of these appearances were greater in mice treated with 100 µg DEPs than in mice treated with 50 µg. In 25 µg DEPs treated groups showed only similar histopathological patterns. However, the lesions were milder and occupied a small area of the lung specimens.

Transmission Electron microscopy: Increased numbers of pinocytic vesicles in alveolar type I cells and

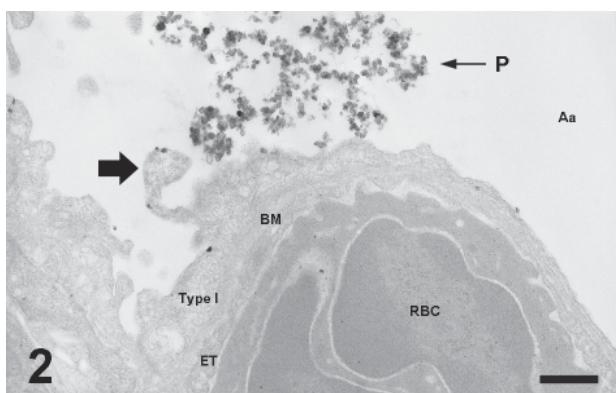


Figure 2. An electron micrograph of the lung from treated mouse revealed desquamation of necrotic alveolar type I cells (solid arrow) with aggregation of DEPs particles; 50 µg treated group, TEM, bar = 2.4 µm. Aa: alveolar air space, BM: basement membrane, ET: alveolar endothelial cell, RBC: red blood cell, Type I: type I alveolar epithelial cell, P: DEPs particles.

endothelial cells of lungs from DEPs treated animals was observed at the area, where the aggregation of DEPs was also present. In 50 and 100 µg DEPs treated groups, type I alveolar epithelial cells that apposed to the clump of particles had features of necrosis and desquamation leading to direct exposure of interstitial tissue to the alveolar space. Damage and dissociation of basement membranes were also found associated with type I alveolar epithelial cell erosion from 5 min onwards (Figure 2).

Time effects of SRM 2975

Histopathology: In the control groups, no significant lesion was observed at all time points. At the acute stages of experiment (from 0 hr to 7 days post-exposure), free singlet and aggregate forms of particles were observed on the bronchiolar epithelial cell surface and along the apical surface of the plasma membrane of alveoli. Many singlet DEPs were seen in alveolar epithelial cell cytoplasm, in which some particles were occasionally observed in alveolar capillary lumens even at 0 min post-exposure. Increasing number of active and particle-laden alveolar macrophages was seen scattering around the accumulation of particles in alveoli and bronchioles. Increasing of cells in alveolar septal walls was also found in the area related to DEPs accumulation. At 12 and 24 hr after instillation, a number of nodular aggregations consisted of neutrophils, active AMs, particle-laden AMs and some cell debris were observed in some alveolar regions adjacent to the

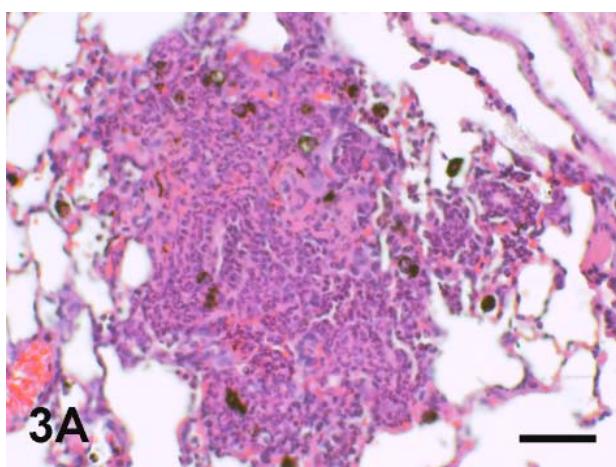
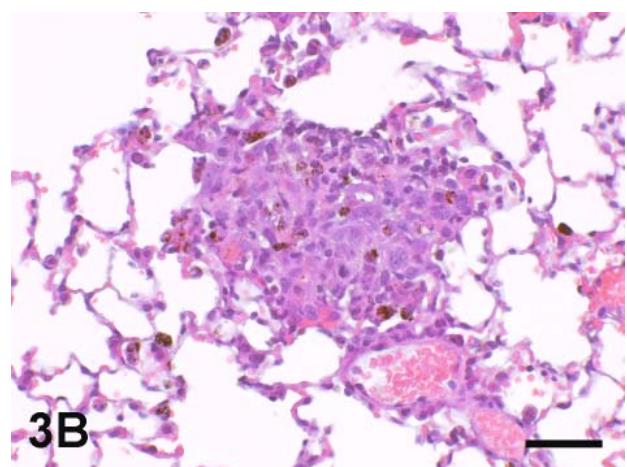


Figure 3. Photomicrographs from the lungs of 50 µg DEPs-treated mice sacrificed at various time points. H&E stain. (A) Treated mice sacrificed at 1 day after exposure showed moderate infiltration of neutrophils with an inflammatory nodule in alveolar air spaces. (bar = 700 µm). (B) Mice sacrificed at 3 days after instillation with DEPs showed moderate focal alveolitis consisting of numerous active AMs, particle-laden AMs, lymphocytes and fewer neutrophils. (bar = 700 µm).



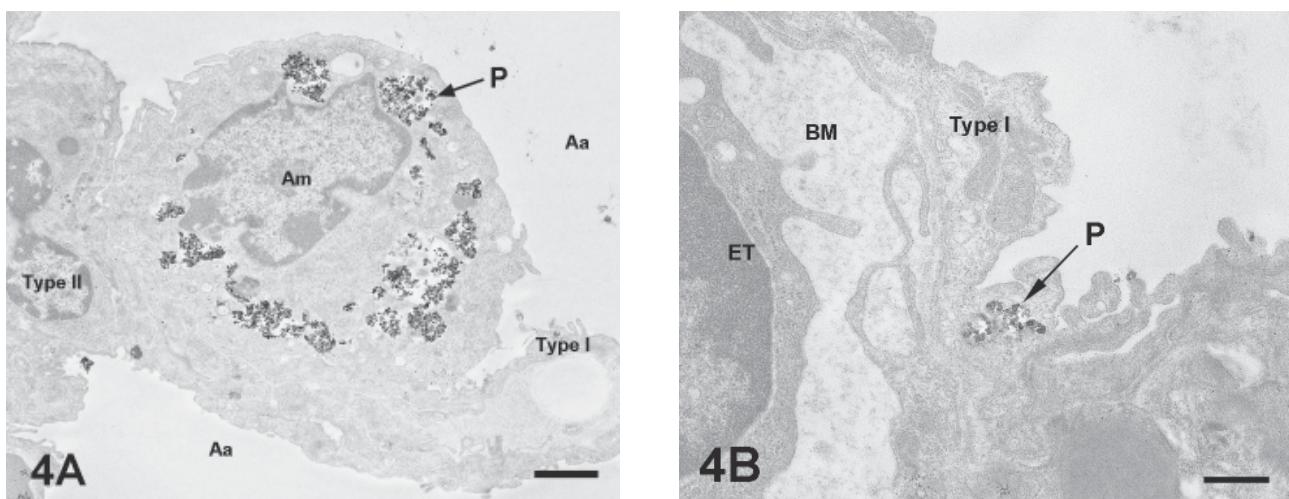


Figure 4. Ultrastructural studies of lungs from various time points of 50 µg DEPs treated groups show the distribution of DEPs in particle-laden alveolar macrophage (A), bar = 1.6 µm. and cytoplasm of alveolar type I cells (B; arrow), bar = 5 µm. Aa: alveolar air space, Am: alveolar macrophage, BM: basement membrane, ET: alveolar endothelial cell, Type I: type I alveolar epithelial cell, Type II: type II alveolar epithelial cell, P: DEPs particles.

bronchioles (Figure 3A). Particle-laden AMs and neutrophils infiltration into bronchial associated lymphoid tissue (BALT) was also observed. By 48 and 72 hr after instillation, moderate focal alveolitis characterized by accumulation of numerous active AMs, particle-laden AMs, lymphocytes and some neutrophils was observed at the terminal bronchiolar and alveolar duct regions (Figure 3B). Changes in the lungs of mice killed at 5 and 7 days after DEPs instillation were restricted to the appearance of the aggregated small foci consisting of particle-laden AMs, active AMs, lymphocytes and fibroblasts with occasional type II epithelial cell regenerative hyperplasia. The hilar lymph nodes were slightly enlarged associated with accumulation of particle-laden macrophages and hyperplastic histiocytes in subcapsular and medullary sinuses. At the subacute stages (from 15 to 30 days post-exposure), the inflammatory foci in lung parenchyma were reduced in number and more focally concentrated.

Transmission Electron microscopy: Numerous free particles, singlet and aggregate forms, of DEPs were observed on the bronchiolar epithelial cell surface and along the apical surface of the plasma membrane of alveoli in treated animals. Accumulation of particle-laden AMs was also found in the alveoli and bronchiolar lumens even at 24 hr post-exposure. These particle-laden AMs showed round or polygonal shapes with numerous lysosomes and phagolysosomes containing particles (Figure 4A). Instilled particles were also observed in the

cytoplasm of both type I and II alveolar epithelial cells (Figure 4B). Increasing number of pinocytic vesicles with some particle-containing in type I alveolar epithelial cells and capillary endothelium was also observed. Necrosis and desquamation of type I alveolar epithelial cells were found particularly in the areas apposing to the clump of DEPs leading to direct exposure of interstitial tissue to the alveolar space. Damage and dissociation of basement membranes were also found associated with type I alveolar epithelial cell erosion.

Discussion

To estimate the suitable doses based on dose-dependent toxicity, we exposed mice with a single intratracheal instillation of various low doses of DEPs. Our data showed that even a low dose (25 µg/mouse) of DEPs can induce pulmonary effects characterized by mild inflammatory cell infiltration, mainly alveolar macrophages and neutrophils, increasing number of cells in alveolar wall and particle-laden AMs accumulation. By contrast, instillation of higher doses (50 or 100 µg) DEPs produced mild to moderate pulmonary inflammation and injury, as evidenced by infiltration of neutrophils and active AMs, focal alveolitis and particle-laden AMs accumulation. Ultrastructural studies in treated animals showed the dissociation of basement membranes and erosion of type I alveolar epithelial cells, which were associated with particle accumulation. These pathological

appearances suggested that DEPs can induce alterations in the permeability of the alveolar-capillary barrier resulted in the disturbance of leakage of the transudation of serum proteins from the vasculature into alveolar lumens similar to the previous study (Singh et al., 2004). From the results of dose response experiments in recent study, 50 μg was suggested to be the most appropriate dose for sensitive detection of acute and subacute inflammatory changes in lungs exposed to DEPs. The dose of 100 μg did not induce any novel extra alterations with a little bit too large to allow assessment of slight changes and the distribution of particles in lung parenchyma. In addition, 25 μg was too weak to cause changes in histological parameters on subacute stage. Ichinose (1995) reported that intratracheal instillation of 400 or 800 μg DEPs per mouse at 24 hr post-exposure produced a moderate to marked pulmonary inflammation and injury effects characterized by influx of neutrophils, accumulation of particle-laden alveolar macrophages, and alveolar type I cell and basement membrane damages that quite similar to our histological changes at acute phase but caused much more severe lesions because of large volume of particles.

The results of time effects showed that instillation of low dose of DEPs (50 μg) induced mild to moderate pulmonary inflammation and injury characterized by infiltration of neutrophils and active AMs, focal alveolitis and particle-laden AMs accumulation at subacute stage. However, the lung lesions with occasional regenerative hyperplasia of type II epithelial cells were milder during subacute phase. Ultrastructural finding showed the dissociation of basement membranes in alveolar area related with the particle accumulation, and then gradually recovered to normal appearances. Chronologically, the lung lesions were getting better by time because of effective clearance system against toxic effects associated with particle exposure. The clearance pathways of insoluble particles deposited in the alveolar region involves phagocytosis by alveolar macrophages followed by mucociliary clearance system and through the lymphatic system (Sun et al., 1984; Cohen et al., 1985; Yu and Rappaport, 1996). From the histological results, we suggested that the most prominent alveolar clearance pathway in our study was mediated by alveolar macrophages, primarily through migration of particle laden

cells to the ciliated airways, and to a small portion through penetration of these cells into the interstitium where they were either retained or transported to the lymphatic system.

There are three hypothetical mechanisms of the particulate matter translocation at the air-blood barrier including cell mediated active transportation (phagocytosis by macrophages or endocytosis by alveolar epithelial cells), passive transportation (diffusion pathway), active or passive transportation through gaps between alveolar epithelial cells (Heckel et al., 2004; Oberdorster et al., 2005). From the results of our ultrastructural study, DEPs could translocate through air-blood barriers to the circulation by cell mediated active transportation, even phagocytosis by macrophages or endocytosis by alveolar epithelial cells. Moreover, damage of alveolar type I cells and basement membranes caused by exposure to DEPs enhance the opportunity of particles to penetrate directly into the blood circulation.

In summary, this study showed pathological details of acute pulmonary inflammation and tissue injury induced by intratracheal instillation of various low doses of DEPs. Dose-response pulmonary effect of DEPs (SRM 2975) was also revealed. In the time effect study, the main translocation of DEPs (SRM 2975) might be translocate through air-blood barriers to the circulation by cell mediated active transportation, either phagocytosis by macrophages or endocytosis by alveolar epithelial cells. Moreover, alveolar basement membrane damages caused by DEPs might be one of the causes of the translocation.

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