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Effects of Carbon Black Nanoparticles on Elastase-Induced Emphysematous Lung Injury in Mice

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Abstract: Although adverse health effects of particulate matter with a diameter of <100 nm (nanoparticles) have been proposed, biological evidence supporting their promotion of the inflammatory lung response *in vivo* is limited. This study investigated the impact of pulmonary exposure to carbon black nanoparticles (CBNP) on emphysematous lung injury induced by porcine pancreatic elastase (PPE) in mice. Vehicle, two sizes (14 and 56 nm) of CBNP (50 μg/body: 4 mg/kg), PPE (0.03 U/body: 1 U/kg) or PPE + CBNP was administered intratracheally; thereafter, parameters of inflammatory lung changes were evaluated at several time-points. CBNP of 14 nm significantly induced acute lung inflammation in non-elicited subjects and aggravated PPE-elicited airway neutrophilic inflammation at an early stage (day 1), which was concomitant with the enhanced lung expression of pro-inflammatory cytokines such as interleukin-1β and chemokine such as keratinocyte-derived chemoattractant. Further, 14-nm CBNP exaggerated emphysematous lung structural changes at a delayed stage (day 14). On the other hand, 56-nm CBNP induced lung inflammation but did not influence PPE-elicited pathophysiology in the lung. Taken together, CBNP at an optimal size and dose can exacerbate PPE-induced pulmonary inflammation and emphysema. This enhancement may be mediated, at least partly, via the increased local expression of pro-inflammatory molecules.

Previous epidemiological studies have indicated that exposure to ambient particulate matter (PM) is linked to increases in mortality and morbidity related to respiratory diseases [1,2]. In particular, the concentration of PM of mass median aerodynamic diameter (a density-dependent unit of measure used to describe the diameter of particles) < or 2.5 μm (PM 2.5) is closely associated with both acute and chronic respiratory effects and subsequent mortality [3]. Among a variety of constituents involved in PM 2.5, diesel exhaust particles (DEP), which are small (mean size: 200–400 nm) with carbonaceous cores [4], are important because of their apparent toxicity in urban areas [5,6]. Reportedly, DEP not only induce respiratory toxicity but also facilitate sensitive lung diseases *in vivo* [7–10].

To date, alternatively, nanoparticles (NP), particles <0.1 μm in mass median aerodynamic diameter, have been postulated to be increasing in the environment and affect cardiopulmonary systems [3,11,12]. Nanoparticles are reportedly able to penetrate deeply into the respiratory tract and have a larger surface area than particles with a larger size, thus resulting in a more marked inflammatory response [13–16]. Furthermore, we previously demonstrated that carbon nanoparticles can aggravate two forms of pulmonary inflammation: allergic airway [17] and endotoxin-related lung inflammation. However, the effects of nanoparticles, particularly their size-related effects, on sensitive pulmonary inflammatory conditions have not been fully investigated.

Chronic obstructive pulmonary disease (COPD) is one of the chronic inflammatory diseases of the lung that is associated with reduced maximal expiratory flow, increased lung volume and alveolar wall destruction [18]. COPD is currently the fourth leading cause of death in the United States, with up to 7 million patients diagnosed each year [19]. It has been predicted by the World Health Organization that the prevalence of COPD will increase in the coming years to become the fifth most common cause of morbidity and the third most common chronic disease worldwide [20]. The disorder is reportedly caused/exacerbated by air pollutants, including cigarette smoke and PM [21]. In fact, PM is epidemiologically implicated to link to the degree of symptoms of COPD [22–24]; however, biological evidence remains unclarified. Emphysema is a major component of COPD and is experimentally reproduced by an intratracheal administration of porcine pancreatic elastase (PPE) to rodents as 'elastase-inducible emphysema models' [25,26]. To date, the effects of PM on the emphysema model have rarely been examined [27]; in particular, the effects of NP have never been investigated.

In this study, we elucidated the effects of a single intratracheal administration of CBNP on PPE-induced pulmonary inflammation and emphysema in mice.

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Materials and Methods

Animals. Male ICR mice 6–7 weeks old (weighing 29–33 g; Japan Clea Co., Tokyo, Japan) were used. The mice were housed in an animal facility maintained at 24–26°C with 55–75% humidity and a 12-hr light: dark cycle and fed a commercial diet (Japan Clea Co.) and given water *ad libitum*.

CBNP. CB particles with primary particle diameter of 14 and 56 nm (Printex 90 and Printex 25, respectively) were obtained from Degussa, Germany. The surface area of the 14-nm CBNP was 300 m^2/g and that of 56-nm CBNP was 45 m^2/g. By using JEM-2010 transmission electron microscope (TEM; JEOL, Tokyo, Japan), the size of each particle was quantified and it was confirmed that both sizes of CBNP were evenly dispersed (distribution), spherical (shape) and mostly singlet particles (aggregation status) [28 and personal data]. Applied number concentration of 14-nm or 56-nm CBNP was 2.44×10^{13} or 2.28×10^{11}, respectively, as estimated previously [28]. CBNP were autoclaved at 250°C for 2 hr before use. The suspension was sonicated for 3 min. using an ultrasonic disrupter (UD-201; Tomy Seiko, Tokyo, Japan).

Study protocol. Mice were divided into six experimental groups. The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Nissui Pharmaceutical Co., Tokyo, Japan) containing 0.05% Tween 80 (Nacalai Tesque, Kyoto, Japan) once. The PPE group received 0.03 U of porcine pancreatic elastase (PPE, 48.0 U/mg protein; CALBIOCHEM, EMD Biosciences Inc., San Diego, CA, USA) dissolved in the same vehicle. The CBNP groups received 50 μg of CBNP suspended in the same vehicle. The PPE + CBNP groups received a combination of PPE with each CBNP in the same vehicle. In each group, vehicle, PPE, CBNP or PPE + CBNP was suspended in 0.1-ml aliquots and inoculated by the intratracheal route through a polyethylene tube under anaesthesia with 4% halothane (Hoechst, Tokyo, Japan). The animals were studied 1, 7 or 14 days after the intratracheal administration, involving bronchoalveolar lavage (BAL), lung histology and protein levels of cytokines and chemokines in the lung homogenates. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by the Institutional Review Board.

BAL. The mice were killed by etherization and exsanguinated from the abdominal aorta. A cannula was inserted into the trachea and secured with a suture. The lungs were lavaged three times with 1.2 ml of sterile saline at 37°C, which was instilled bilaterally with a syringe. The fluid was harvested by gentle aspiration. The collected fluid was cooled and centrifuged at $300 \times g$ for 10 min. Total cell counts were made using a Burker-Tulk counting chamber. Differential cell counts were assessed in cytological preparations. Slides were prepared using an Autosmear (Sakura Seiki Co., Tokyo, Japan) and stained with Diff-Quik (International reagents Co., Kobe, Japan). A total of 500 cells were counted under oil immersion microscopy (n = 8 in each group).

Lung morphometry. In a separate series of experiments, the mice were killed by etherization and exsanguinated from the abdominal aorta. Both lungs were fixed with 10% buffered formalin infused through the trachea at a pressure of 20 cm H_2O. After separation of the lobe, 2-mm-thick blocks were taken for paraffin embedding. Three-micrometre-thick sections were stained with haematoxylin and eosin to observe and evaluate the degree of infiltration of eosinophils, neutrophils and mononuclear cells as well as the lung structure. The mean linear intercept (Lm), an indicator of the air space size, was calculated for each mouse from 10 randomly selected fields at a magnification of × 200 (n = 5–6 in each group) as previously described [29].

Quantitation of the levels of cytokines and chemokines in lung homogenates. Lung homogenate supernatants were prepared as follows. After BAL, the lungs were removed and subsequently homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St. Louis, MO, USA), 0.1 mM phenylmethanesulfonyl fluoride (Nacalai Tesque, Kyoto, Japan), 1 μM pepstatin A (Peptide Institute, Osaka, Japan) and 2 μM leupeptin (Peptide Institute), as described previously. The homogenates were then centrifuged at $105{,}000 \times g$ for 1 hr. Thereafter, the supernatants were stored at −80°C. ELISA for interleukin (IL)-1β (R&D Systems, Minneapolis, MN), IL-6, IL-13, interferon (IFN)-γ, tumour necrosis factor (TNF)-α (Endogen, Cambridge, MA, USA), macrophage chemoattractant protein (MCP)-1 (R&D Systems) and keratinocyte-derived chemoattractant (KC: R&D Systems) were conducted in the lung homogenates according to the manufacturer's instructions.

Statistical analysis. Data are shown as the mean ± S.E.M. Data were analysed by one-way or two-way ANOVA to examine whether there was any statistical difference among the experimental groups (Stat view version 5.0; Abacus Concepts Inc., Berkeley, CA, USA). If the difference was significant, Bonferroni post-tests or Tukey's multiple comparison test was used for paired comparison. Values of $p < 0.05$ were considered significant.

Results

The effects of CBNP on airway inflammation in a PPE-induced murine emphysema model.

At first, we employed various doses of CBNP (1–50 μg/body) and PPE (0.03–0.3 U/body) to identify a pair having a significant impact on airway inflammation in this model on analyses of BAL cellularity. As a result, data performed with CBNP (50 μg) and PPE (0.03 U) revealed the largest difference between PPE and PPE + CBNP groups; thus, we decided to select such doses (fig. 1). On day 1 (fig. 1A), exposure to PPE plus 14-nm CBNP significantly increased the total number of cells when compared with that to vehicle or 14-nm CBNP ($p < 0.05$). The neutrophil number was significantly greater in the 14-nm CBNP ($p < 0.01$), 56-nm CBNP ($p < 0.05$), PPE ($p < 0.05$), PPE + 14-nm CBNP ($p < 0.01$) or PPE + 56-nm CBNP ($p < 0.05$) groups than in the vehicle group. The number was even greater in the PPE + 14-nm CBNP group than in the PPE ($p < 0.01$) or 14-nm CBNP ($p < 0.05$) group. On the other hand, the macrophage number was significantly lower in the 14-nm CBNP than in the vehicle group ($p < 0.05$), and PPE + 14-nm CBNP than in the PPE group ($p < 0.01$). On day 7 (fig. 1B), exposure to 14-nm CBNP and PPE significantly increased the number of total cells and macrophages when compared with that to vehicle ($p < 0.01$). The neutrophil number was significantly higher in the PPE + 14-nm CBNP or PPE + 56-nm CBNP group than in the vehicle group ($p < 0.05$). The number was also higher in the PPE + 14-nm CBNP than in the PPE group ($p < 0.05$).

The effects of CBNP on lung structural changes in the presence or absence of PPE.

We evaluated lung specimens that had been stained with haematoxylin and eosin 14 days after the intratracheal administration of PPE (fig. 2) according to our previous report [29]. Light microscopic examination revealed that the intratracheal instillation of CBNP led to the diffuse deposition of

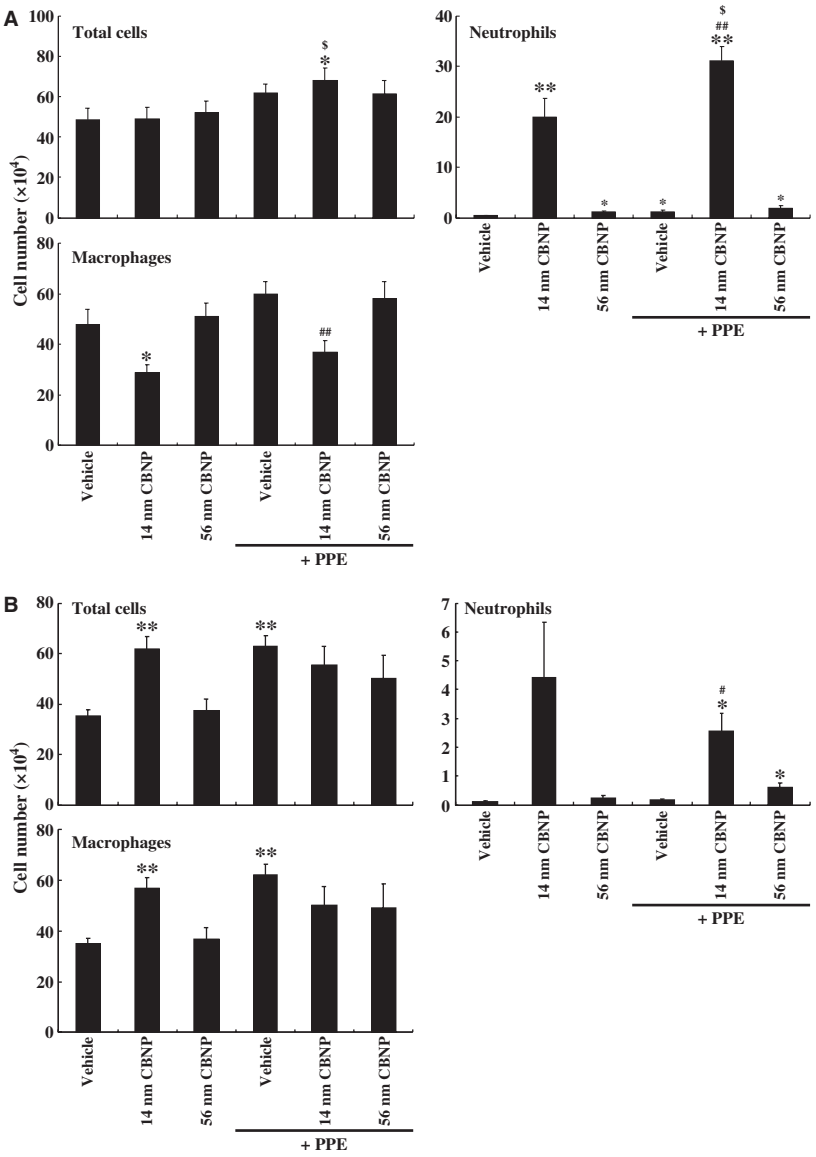

Fig. 1. Effects of carbon black nanoparticles (CBNP) on airway inflammation in the murine emphysema model on days 1 (A) and 7 (B). (A, B) Total cell, neutrophil and macrophage counts of BAL cells from each mouse. BAL was performed 1 and 7 days after the intratracheal administration of the vehicle, porcine pancreatic elastase (PPE) (0.03 U/body), CBNP (50 μg/body) or PPE + diesel exhaust particles (DEP). Differential cell counts were performed on 500 cells. Results are means ± S.E. (n = 8 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* the vehicle group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *versus* the PPE group. $^{\$}p < 0.05$ *versus* the corresponding CBNP group.

particles in the bilateral lungs, including the bronchi and alveolar spaces (mainly observed in phagocytosing macrophages: data not shown). As for inflammatory leucocyte infiltration in the lung parenchyma, exposure to CBNP, PPE or PPE + CBNP induced moderate inflammation of the lung parenchyma, characterized by the infiltration of polymorphonuclear leucocytes into the inflamed sites. At this stage, however, the inflammatory change did not differ between the experimental groups (CBNP, PPE and PPE + CBNP groups). Vehicle administration caused the limited infiltration of inflammatory cells. Regarding emphysematous change, the lungs treated with CBNP showed no apparent emphysematous alteration. On the other hand, the lungs treated with PPE or PPE + CBNP revealed emphysematous changes such as airspace enlargement and the progressive destruction of alveolar wall structures (fig. 2A). However, there were no clear differences in morphological changes between the PPE and PPE + CBNP groups, although the change in the PPE + CBNP groups, particularly in the PPE + 14-nm CBNP group, seemed to be more marked than that in the PPE group. Semi-quantitative analysis (fig. 2B) showed that Lm was significantly greater in the PPE or PPE + CBNP groups than in the vehicle group ($p < 0.01$). The value was significantly greater in the PPE + 14-nm CBNP group than in the 14-nm CBNP group ($p < 0.05$). Although the value was greater in the PPE + CBNP groups, particularly in the PPE + 14-nm CBNP group, than in the PPE group, the difference was not significant.

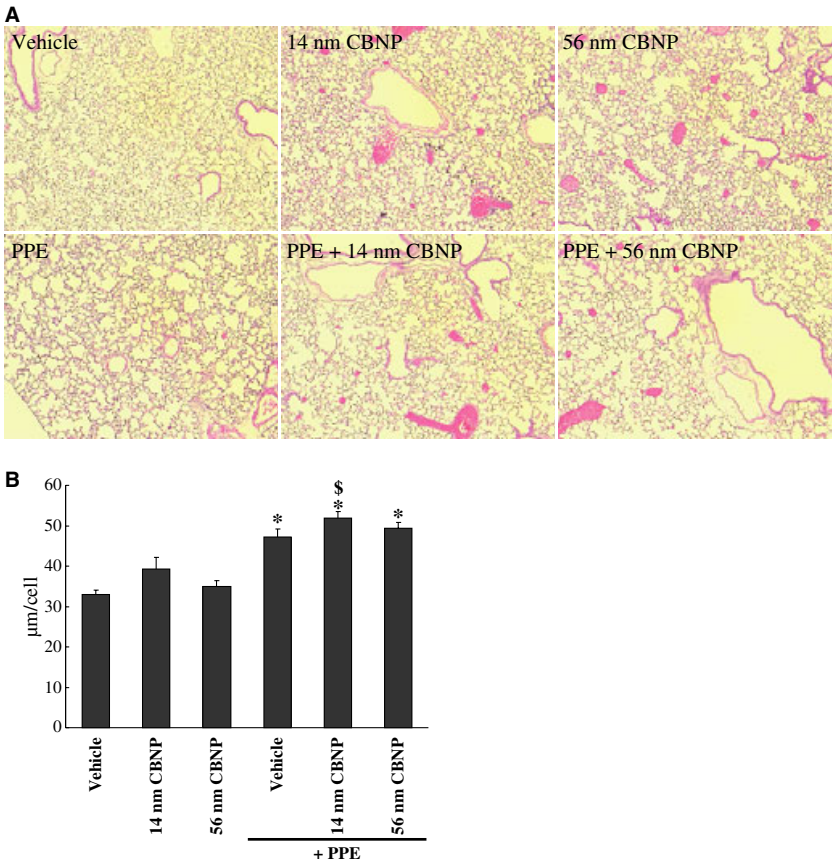

Fig. 2. Effects of carbon black nanoparticles (CBNP) on lung histological changes in the emphysema model. (A) Representative photographs of lung tissue obtained 14 days after the intratracheal administration of the vehicle, CBNP (14 or 56 nm), porcine pancreatic elastase (PPE) or PPE + CBNP (14 or 56 nm) stained with haematoxylin and eosin. Original magnification × 100. (B) Semiquantitative analysis of lung tissue performed with the mean linear intercept, a morphometric parameter of pulmonary emphysema. Data are means ± S.E. (n = 5–6 in each group). $^{*}p < 0.01$ *versus* the vehicle group. $^{\$}p < 0.05$ *versus* the corresponding CBNP group.

The effects of CBNP on cytokine expression in the lung in the presence or absence of PPE.

We examined the levels of proinflammatory cytokines and chemokines in lung tissue homogenates 1 day after the intratracheal administration of PPE (table. 1). The TNF-α level was significantly greater and the levels of IFN-γ and IL-6 were significantly lower in the PPE + 56-nm CBNP than in the 56-nm CBNP group. The administration of 14-nm CBNP and PPE + 14-nm CBNP significantly elevated the IL-1β level when compared to vehicle ($p < 0.01$). Further, the level was greater in the PPE + 14-nm CBNP than in the PPE group ($p < 0.05$). The administration of 14-nm CBNP

Table 1.

Cytokine and chemokine profiles in the lung after intratracheal challenge.

Group	TNF-α	IFN-γ	IL-1β	IL-6	IL-13	KC	MCP-1
	(pg/lung homogenates)						
Vehicle	1318.6 ± 133.5	8603.2 ± 702.7	210.4 ± 56.4	73.41 ± 11.59	N.D.	0 ± 0	22.85 ± 5.23
14 nm CBNP	1246.6 ± 87.8	8291.6 ± 509.3	708.8 ± 100.6*	76.79 ± 7.41	N.D.	76.59 ± 17.88*	71.25 ± 9.40**
56 nm CBNP	1202.1 ± 39.9	8890.0 ± 382.1	272.0 ± 52.8	103.5 ± 9.15	N.D.	2.94 ± 2.94	21.10 ± 6.82
PPE	1619.0 ± 111.3	8189.4 ± 567.2	210.1 ± 60.4	59.69 ± 8.34	N.D.	25.21 ± 13.63	24.38 ± 8.42
PPE + 14 nm CBNP	1391.6 ± 52.8	8047.3 ± 275.9	840.0 ± 81.1*#	72.97 ± 5.86	N.D.	114.2 ± 13.8*#$	76.12 ± 10.21**$$
PPE + 56 nm CBNP	1562.1 ± 94.9$$	7958.2 ± 220.2$	308.2 ± 116.0	63.46 ± 10.60$	N.D.	5.56 ± 4.18	27.37 ± 3.06

Lung tissue samples from each mouse were harvested 1 day after the intratracheal administration of porcine pancreatic elastase (PPE). The levels of tumor necrosis factor (TNF)-α, interferon-γ, interleukin (IL)-1β, IL-6, IL-13, keratinocyte-derived chemoattractant (KC), and macrophage chemoattractant protein (MCP)-1 in the lung homogenate supernatants were measured by ELISA. The results are means ± S.E. (n = 8 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$ *versus* the vehicle group. $^{\#}p < 0.01$ *versus* the PPE group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ *versus* the corresponding carbon black nanoparticles (CBNP) group.

and PPE + 14-nm CBNP significantly elevated the KC level when compared to vehicle ($p < 0.01$). The level was also greater in the PPE + 14-nm CBNP group than in the PPE or 14-nm CBNP group ($p < 0.05$). The MCP-1 level was also significantly greater in the 14-nm CBNP or the PPE + 14-nm CBNP group than in the vehicle group ($p < 0.01$). The level was also greater in the PPE + 14-nm CBNP group than in the PPE group ($p < 0.01$). The IL-13 value was below the detection limit in all the experimental groups.

Discussion

The present study showed that a single intratracheal administration of 14-nm CBNP at a dose of 50 μg exacerbates PPE-induced murine airway inflammation, characterized by leucocyte reflux into the airway in the acute phase (day 1) and moderately subsequent devastating lung structural (emphysematous) changes in the late-phase (day 14), whereas 56-nm CBNP did not have such effects. In the acute phase (day 1), CBNP (14 nm) increased the lung expression of a pro-inflammatory cytokine (IL-1β) and chemokine (KC) in the presence or absence of PPE.

A host inflammatory or immune response to inhaled particles might lead to pulmonary emphysema and COPD in human beings [30]. Indeed, the intratracheal administration of titanium dioxide nanoparticles reportedly induces emphysema-like lesions in the lung *in vivo* [31]. Further, pulmonary emphysema is epidemiologically [24,32–34] and experimentally [27] susceptible to PM. However, no biological study has examined the aggravating effect of nanoparticles on pre-existing protease-induced lytic pulmonary emphysema. Furthermore, there was no study comparing the effects on the model system between several sizes of PM. In the present study, we first examined combined challenge with 1–50 μg/animal of CBNP and 1–10 U/kg PPE to investigate several experimental situations and identify a dose pairing showing overt facilitation on the pathophysiology (data not shown). As a result, 50 μg of CBNP and 0.03 U of PPE caused the most marked deterioration of the pathology. However, no marked synergistic pattern was observed in the PPE + CBNP group in this experimental setting, as was noted in allergic airway inflammation and infectious lung injury models [17,35]. Possible explanations for this phenomenon may include the animal strains/species, pathological conditions (type and/or degree of inflammation) and/or CBNP exposure protocols (route, dose, timing, duration and/or end-point). In fact, for example, Lopes and colleagues recently showed that chronic (2 months) exposure to an ambient level (mean concentration: 34 $\mu g/m^3$) of PM 10 (emitted by traffic) worsens murine emphysema induced by papain [27]. Thus, other protocols should be examined in the future to clarify/validate the effects of acute, subacute or even chronic exposure to CBNP on the induction/progression of pulmonary emphysema *in vivo*.

Our present study is interesting in that two different types of CBNP differently affected the PPE-induced emphysematous lung injury. In our study, the surface area of the 14-nm CBNP was 6.7-fold larger than that of 56-nm CBNP (300 *versus* 45 m^2/g, respectively). These results were consistent with our previous findings regarding other lung inflammation models, i.e. allergic airway inflammation and infectious lung injury related to endotoxin. Nanoparticles with a larger surface area are likely to attach more immunoregulative molecules than those with a smaller surface area. Alternate, we cannot exclude the possibility that a significant characteristic difference existed, such as the metal concentration between the two CBNP, which might be responsible for different outcomes. As well, we did not examine the effects of nanoparticles with the same particle number in the present study. Generally, the particle number of a smaller particle is greater than that of a larger one when the particles are of the same weight as found in our present study. Alternatively, therefore, our study has demonstrated not only the size-related effects of nanoparticles but also the effects of their surface area and/or numbers on PPE-induced lung inflammation. Future independent studies involving standardization of the surface area or particle number will facilitate a better understanding of the effects of nanoparticles on the pathophysiology.

KC, a murine homologue of IL-8, is reportedly involved in the pathogenesis of COPD [36]. In smokers, IL-8, as well as ICAM-1, was up-regulated in epithelial cells [37]. In contrast, it was reported that macrophage-derived IL-8 does not correlate with the COPD pathology [38]. IL-1β also increases in smokers, and, experimentally, is important for disease progression [39,40]. On the other hand, alveolar macrophages play an important role in the initial defence against various environmental particles via several scavenger receptors [41]. In the present study, KC and IL-1β levels in the lung were greater in the PPE + CBNP group than in the PPE or CBNP group. Therefore, CBNP might promote pulmonary emphysema, at least in part, through the enhanced lung expression of these chemokines and cytokines. Alternatively, CBNP may be a risk for COPD in human beings in the context of the promoting properties of these pro-inflammatory molecules in the lung. Nonetheless, the cellular contribution (i.e. epithelial cells, alveolar macrophages or other cell types) remains to be elucidated in the future.

Recently, we have shown that a single intratracheal administration of DEP at a dose of 200 μg does not facilitate this type of murine pulmonary emphysema [42]. The study also provides a hint for the concept that smaller particles (nanoparticles) can render greater adverse effects on the pathophysiology than larger ones, because the average size of DEP is 400 nm [8,10]. However, in that study, we applied a larger volume of PPE (1 U/animal) than the present one. Furthermore, there might be significant differences in the characteristics of the particles, such as physicochemical properties, electronic charge and aggregation rate. Therefore, additional investigations are needed in the future to confirm size impact of the PM.

In conclusion, CBNP of an optimal size and dose can exacerbate PPE-induced pulmonary inflammation and emphysema. This enhancement may be mediated, at least

partly, via the increased local expression of proinflammatory molecules. These results suggest/implicate that inhalable nanoparticles become an important environmental risk factor for emphysematous lung inflammatory disorders such as COPD in the future.

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