Effects of Pulmonary Exposure to Carbon Nanotubes on Lung and Systemic Inflammation with Coagulatory Disturbance Induced by Lipopolysaccharide in Mice

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Despite intensive research as to the pathogenesis of lipopolysaccharide (LPS)-related inflammation with coagulatory disturbance, their exacerbating factors have not been well explored. This study examined the effects of pulmonary exposure to two types of nano-sized materials (carbon nanotubes: CNT [single-wall: SWCNT, and multi-wall: MWCNT]) on lung inflammation and consequent systemic inflammation with coagulatory disturbance induced by pulmonary exposure to LPS in mice and their cellular mechanisms *in vitro*. ICR male mice were divided into 6 experimental groups that intratracheally received the vehicle, two types of CNT (4 mg/kg), LPS (33 μg/kg), or LPS plus either type of CNT. Twenty-four hours after treatment, both types of CNT alone induced lung inflammation with enhanced lung expression of proinflammatory cytokines, but did not synergistically exacerbate lung inflammation elicited by LPS. SWCNT significantly induced/enhanced pulmonary permeability and hyperfibrinogenemia and reduced activated protein C in the absence or presence of LPS, whereas MWCNT did moderately. Both CNT moderately, but not significantly, elevated circulatory levels of proinflammatory cytokines and chemokines. In the presence of LPS, CNT tended to elevate the levels of the mediators with an overall trend, which was more prominent with SWCNT than with MWCNT. *In vitro* study showed that both CNT amplified LPS-induced cytokine production from peripheral blood monocytes. These results suggest that CNT can facilitate systemic inflammation with coagulatory disturbance, at least in part, via the activation of mononuclear cells, which is accompanied by moderate enhancement of acute lung inflammation related to LPS. Exp Biol Med 233:1583–1590, 2008

Key words: carbon nanotubes; lipopolysaccharide; lung inflammation; systemic inflammation; coagulatory disturbance

Introduction

Endotoxin (lipopolysaccharide: LPS) induces systemic inflammatory response syndrome (SIRS), such as acute lung and systemic inflammation/injury and multiple organ dysfunction, frequently accompanied by consumptive hemostatic changes and often leading to coagulopathy including disseminated intravascular coagulation (DIC) (1). Although intensive research into the pathogenesis of SIRS with or without DIC and other coagulopathy has been conducted, their exacerbating factors have not yet been explored. Epidemiologically, environmental particulate matters of mass median aerodynamic diameter (a density-dependent unit of measure used to describe the diameter of the particle) $<$ or 2.5 μm ($PM_{2.5}$), including diesel exhaust particles (DEP) and nanoparticles ($<$100 nm) relate to the induction/enhancement of cardiopulmonary diseases, including lung inflammation, allergic asthma, pulmonary emphysema, and ischemic (coronary) heart diseases (2–9). On the other hand, LPS is a significant constituent of many air pollutant particles and has accordingly been implicated in PM effects (10). To support these reports, we have previously demonstrated that DEP, their components (especially residual particles after extraction of chemical

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components: washed DEP), and carbon nanoparticles enhance not only acute lung injury but systemic inflammation with coagulatory disturbance related to LPS (6, 11–13). Taken together, these findings demonstrate that environment-existing particles have aggravating effects on both lung and systemic inflammation with coagulopathy related to LPS.

The development of nano-technology has increased the risk of our exposure to types of particles other than combustion-derived particles in the environment, namely engineered nanomaterials (14). As these materials become more widespread, many questions arise as to the consequences that nanomaterials may have on the environment. In fact, previous reports showed that the full impact, or even partial impact, of manufactured nanomaterials on health and the environment has yet to be explored in depth (15–19). Among nanomaterials, carbon nanotubes (CNT) are unique. CNT possess significant characteristics in size (1–20 nm width, and many microns in length), strength, and surface chemistry (20). Notably, their length/width (aspect) ratios of >1000, reactive surface chemistry, and/or poor solubility raise concerns linked to past experience with hazardous fibers, including asbestos. CNT exist in many forms, including single-walled CNT (SWCNT), double-walled, and multi-walled (MWCNT). Both SWCNT and MWCNT are being applied in wide-ranging areas from the semiconductor industry to drug delivery and super-light and strong composite materials (21) and are reportedly increasing in environmental exposure (22). The aim of the present study was therefore to experimentally elucidate whether two types of CNT (SWCNT and MWCNT) exacerbate lung and systemic inflammation with coagulatory disturbance induced by intratracheal administration of LPS in mice. In addition, effects of CNT on the production/release/expression of cytokines (tumor necrosis factor [TNF]-α and interleukin [IL]-1β) and chemokines (macrophage inflammatory protein [MIP]-1α, macrophage chemoattractant protein [MCP]-1, and keratinocyte-derived chemoattractant [KC]), which are reportedly induced by LPS and play an important role in local and systemic inflammation and/or coagulopathy both *in vivo* and *in vitro* (23, 24) to seek cellular mechanisms.

Materials and Methods

Animals. Male ICR mice, 6 weeks of age and weighing 28 to 33 g (Japan Clea Co., Tokyo, Japan) were used in all experiments. They were fed a commercial diet (Japan Clea Co.) and given water ad libitum. The mice were housed in an animal facility that was maintained at 24°C to 26°C with 55% to 75% humidity and a 12-h light/dark cycle.

CNT. Both SWCNT and MWCNT were purchased from SES Research (TX, USA). SWCNT were formed in a carbon arc in the presence of a metal catalyst. SWCNT ranged from 1.2–1.4 nm in diameter and 2–5 μm in length. MWCNT ranged from 2–20 nm in diameter, and 100 nm to several microns long with 5–20 graphitic layers. Both nanotubes were treated to open the ends of the nanotubes and to remove the amorphous carbon and nanoparticles. The end products contained up to 75% pure nanotubes and a larger percentage of open-ended tubes with the remaining material consisting of amorphous carbon and other carbon nanoparticles.

Study Protocol. Mice were divided into six experimental groups. The vehicle group received phosphate-buffered saline (PBS) at pH 7.4 (Invitrogen Co., Carlsbad, CA, USA) containing 1% Tween 80 (Nakalai Tesque, Tsukuba, Japan). The LPS group received 33 μg/kg of LPS (*E. coli*. B55:05, Difco Lab, Detroit, MI) dissolved in the vehicle. The CNT groups received 4 mg/kg of CNT (SWCNT or MWCNT) suspended in the same vehicle. The LPS + CNT groups received combined treatment using the same protocols as the LPS and CNT groups ($n = 20$–25 in each group). CNT were autoclaved at 250°C for 2 h before use, and the suspension was sonicated for 3 min using an Ultrasonic Disrupter (UD-201; Tomy Seiko, Tokyo, Japan). LPS activity, which was determined by Limulus Amebocyte Lysate assay (Seikagaku-kogyo, Tokyo, Japan), was lower than the detection limit (0.001 EU/ml) in the CNT after treatment. In each group, vehicle, CNT, LPS, or LPS + CNT were dissolved in 0.1-ml aliquots, and inoculated by the intratracheal route through a polyethylene tube under anesthesia with 4% halothane (Hoechst, Japan, Tokyo, Japan) as previously described (5, 6, 11–13, 25). The animals were studied 24 h after intratracheal administration. After deep anesthesia, the chest and abdominal walls were opened, and blood was retrieved by cardiac puncture. Blood samples were collected from the right ventricle with direct centrifugation for serum samples or into 3.8% sodium citrate in a ratio of 10:1 with centrifugation for plasma samples. After collection, bronchoalveolar lavage (BAL), pulmonary vascular permeability of water and protein, and protein levels of cytokines and chemokines in the lung tissue supernatants were examined. 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, Pulmonary Vascular Permeability, Lung Histology, and Quantitation of Cytokine and Chemokine Protein Levels in Lung Tissue Supernatants. BAL and cell counts were conducted as previously described ($n = 8$ in each group) (5, 6, 11, 12). In a separate series of experiments, the lungs were weighed and dried as previously reported (3). The wet lung weight – the dry lung weight/body weight was calculated ($n = 8$ in each group). In another experiment, protein concentrations in BAL fluid were determined using the commercially available Bradford protein assay (Bio-Rad Laboratory Inc., Hercules, CA; $n = 8$ in each group) with bovine serum albumin as the standard. In another experiment, the lungs were fixed and stained with hematoxylin and eosin as previously described ($n = 4$–5 in

Table 1. The Number of Total Cells and Neutrophils, Protein Concentration in Bronchoalveolar Lavage (BAL) Fluid, and Lung Water Content After Intratracheal Challenge

Treatment	Total cells	Neutrophils	Protein	Lung wet weight − dry weight [mg]/body weight [g]
Vehicle	52.2 ± 3.6	1.6 ± 0.7	0.68 ± 0.04	4.44 ± 0.10
SWNT	88.4 ± 21.8*	35.8 ± 23.6**	1.55 ± 0.32**	6.46 ± 1.10**
MWCNT	68.8 ± 9.3	22.3 ± 5.9**	0.81 ± 0.05	4.43 ± 0.14
LPS	346.1 ± 35.6**	272.3 ± 26.9**	1.48 ± 0.07**	5.65 ± 0.18**
LPS + SWCNT	373.8 ± 40.4**,$	310.7 ± 31.2**,$	2.59 ± 0.30**,#,$	7.41 ± 0.56**,#
LPS + MWCNT	356.4 ± 57.7**,$	299.2 ± 48.7**,$	1.73 ± 0.16**,$	6.03 ± 0.15**,$

* $P < 0.05$ vs. the vehicle group, ** $P < 0.01$ vs. the vehicle group, # $P < 0.01$ vs. the LPS group, $ $P < 0.01$ vs the corresponding carbon nanotube (CNT) group.

each group) (5, 6, 13). ELISA for IL-1β, MIP-1α, MCP-1, and KC (R&D Systems, Minneapolis, MN, USA) in the lung tissue supernatants were conducted as previously described (6, 13), and the values were expressed as pg/total lung supernatants ($n = 8$ in each group).

Assays for Plasma Levels of Fibrinogen, von Willebrand Factor (vWF), Cytokines, Chemokines, and Activity of Protein C (APC). Plasma levels of fibrinogen and vWF, and the APC ($n = 8$ in each group) were determined using commercial kits (Diagnostica Stargo, Roche, Tokyo, Japan) on STA Compact (Diagnostica Stargo, Roche) as described previously (26). Serum samples ($n = 8$ in each group) were analyzed by ELISA for TNF-α, IL-1β, MIP-1α, MCP-1, and KC (R&D Systems) according to the manufacturer's instructions, and the values were expressed as pg/ml.

Isolation, Culture, and Stimulation of Alveolar Macrophages (AM) and Peripheral Blood Monocytes (PBM). In another experiment, AM were isolated from ICR mice by BAL as previously described (27). All of the lavage fluid cells were pooled and spun at 1,200 *g* for 10 min. Lavage cells were pooled and counted after lysis of red blood cells with hypotonic solution. Heparinized blood was also obtained from ICR mice. All of the blood samples were pooled. Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Viability always exceeded 95% according to trypan blue exclusion. Cells were resuspended in RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) to give a concentration of 1×10^6 cells/ml. 20,000 cells were placed in each well of a 96-well culture plate (IWAKI microplate, ASAHI TECHNO GLASS, Funabashi, Japan) and washed with 200 μl of RPMI 1640 after 2 h, enabling the isolation of AM and PBM by adherence. CNT (1 μg/ml) with or without LPS (10 μg/ml) was then added to selected wells and incubated for 20 h. Culture supernatants were collected and the production of TNF-α, IL-1β, and KC was determined by ELISA as described above ($n = 3$ in each group). The experiment was repeated twice.

Statistical Analysis. Data are reported as the means ± SE. Differences between groups were determined using analysis of variance (ANOVA: Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA, USA) with Bonferroni comparison test. Differences were considered significant if $P < 0.05$.

Results

Effects of CNT on Airway Inflammation and Pulmonary Vascular Permeability. To investigate the magnitude of airway inflammation, we examined the cellular profile of BAL fluid 24 h after intratracheal instillation (Table 1). CNT alone increased the number of total cells and neutrophils as compared with vehicle ($P < 0.05$ for total cells with SWCNT, $P < 0.01$ for neutrophils). LPS exposure significantly increased the numbers as compared with vehicle exposure ($P < 0.01$). The numbers were greater in LPS + CNT groups than in the LPS group (NS) or the corresponding CNT groups ($P < 0.01$). To estimate pulmonary vascular permeability, we examined the protein levels in BAL fluid and the lung water content 24 h after intratracheal instillation (Table 1). Pulmonary exposure to SWCNT significantly elevated the protein levels of BAL fluid and the lung water content as compared to exposure to vehicle ($P < 0.01$). These values were significantly greater in the LPS group ($P < 0.01$) than in the vehicle group. These values were further greater in LPS + CNT groups than in the LPS group ($P < 0.01$ with SWCNT) or the corresponding CNT groups ($P < 0.01$, except for lung water content with LPS + SWCNT; NS).

Effects of CNT on Histological Changes in the Lung. To determine the effects of CNT on lung histology, we evaluated lung specimens stained with hematoxylin and eosin 24 h after intratracheal instillation (Fig. 1). No pathological change was seen in lungs obtained from the vehicle group. Infiltration of neutrophils was moderately seen in the lungs from CNT groups. Treatment with LPS or combined treatment with LPS and CNT showed marked infiltration of neutrophils into the lung parenchyma. Furthermore, the degree and extent were slightly greater in LPS + CNT groups than in the LPS group.

Effects of CNT on the Expression of Proinflammatory Cytokine and Chemokines in the

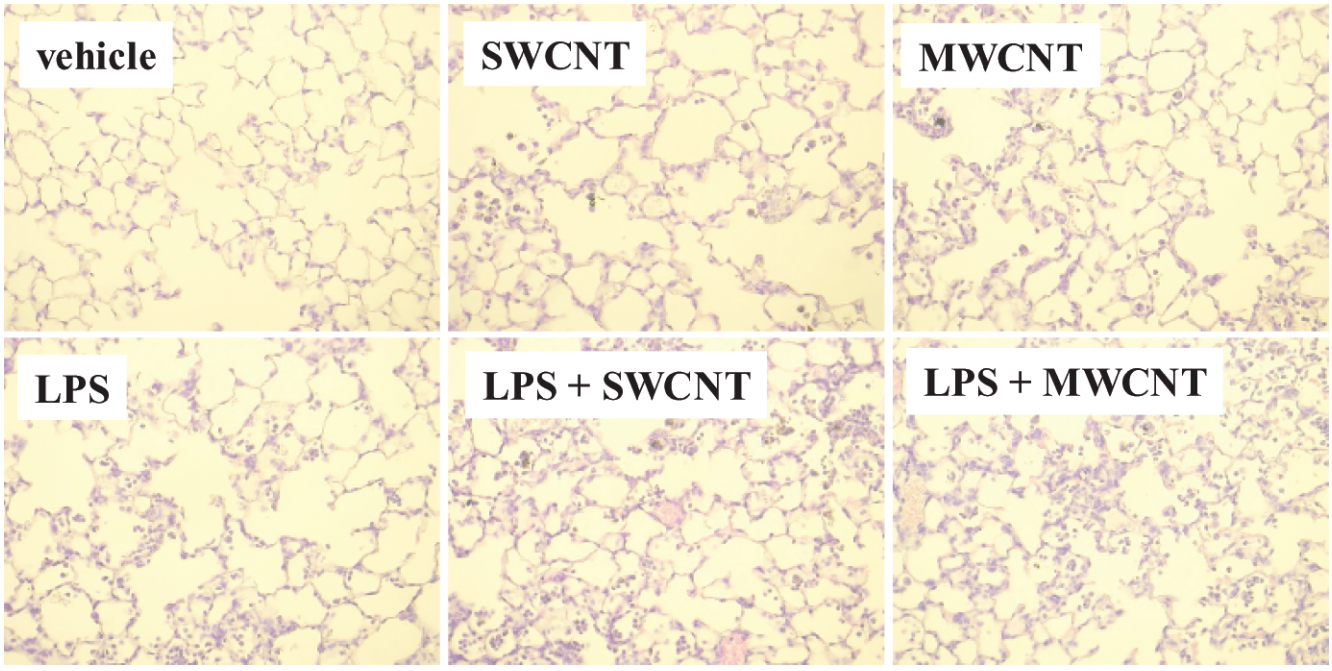

Figure 1. Histopathological findings of the lung. Twenty-four hours after intratracheal administration of vehicle, carbon nanotubes (CNT [single-wall: SWCNT, and multi-wall: MWCNT]), lipopolysaccharide (LPS: 33 μg/kg), or LPS + CNT, mice were killed and lung histology was assessed by hematoxylin and eosin stain ($n = 4$–5 in each group). Original magnification, ×400.

Lung. To investigate the effects of CNT on local cytokine and chemokine expression, we next measured the protein levels of IL-1β, MIP-1α, MCP-1, and KC in lung tissue supernatants 24 h after intratracheal instillation (Table 2). Pulmonary exposure to CNT alone significantly elevated the levels of IL-1β, MIP-1α, and MCP-1 as compared to exposure to vehicle ($P < 0.01$). On the other hand, LPS challenge significantly elevated the levels of all of the proteins as compared with vehicle challenge ($P < 0.01$). These levels were significantly greater in LPS + CNT groups than in the corresponding CNT groups ($P < 0.01$). The IL-1β level was comparable among the LPS and LPS + CNT groups. The other levels were greater in the LPS + CNT groups than in the LPS; however, the difference did not reach statistical significance except for KC with the LPS + MWCNT ($P < 0.05$).

Effects of CNT on Coagulatory Changes. To elucidate the impact of pulmonary exposure to CNT on the coagulatory system, we next analyzed coagulatory parameters 24 h after intratracheal challenge (Table 3). SWCNT exposure significantly elevated the fibrinogen level as compared to vehicle exposure ($P < 0.01$). LPS challenge significantly elevated the values as compared to vehicle challenge ($P < 0.05$). The values were further greater in LPS + CNT groups than in the LPS group ($P < 0.01$ with LPS + SWCNT) or the corresponding CNT groups ($P < 0.01$). As compared to vehicle challenge, LPS, SWCNT, and LPS + SWCNT challenge caused an increase in the level of vWF without statistical significance. CNT, LPS, and LPS + MWCNT decreased APC as compared with vehicle ($P < 0.01$ with SWCNT and LPS + CNT). The activity was further decreased in the LPS + SWCNT group than in the LPS group ($P < 0.01$) or the corresponding CNT group (NS).

Effects of CNT on Circulatory Levels of Proinflammatory Cytokine and Chemokines. To investi-

Table 2. Protein Levels of Interleukin (IL)-1β and Chemokines in the Lung Tissue Supernatants After Intratracheal Challenge

Treatment	IL-1β	MIP-1α	MCP-1	KC
Vehicle	182.6 ± 22.2	2.1 ± 1.2	12.0 ± 2.1	129.5 ± 78.7
SWCNT	1248.6 ± 556.2*	67.7 ± 27.2*	128.1 ± 46.0*	207.9 ± 112.4
MWCNT	673.8 ± 184.3*	53.1 ± 12.6*	53.5 ± 7.3*	244.1 ± 76.6
LPS	6320.0 ± 342.7*	788.8 ± 101.6*	659.8 ± 129.5*	1246.8 ± 147.2*
LPS + SWCNT	6132.3 ± 547.2*,$	946.5 ± 109.5*,$	791.8 ± 180.1*,$	1370.8 ± 193.4*,$
LPS + MWCNT	5390.9 ± 339.6*,$	948.8 ± 146.4*,$	948.9 ± 166.2*,$	1735.6 ± 276.0*,#,$

* $P < 0.01$ vs. the vehicle group, # $P < 0.05$ vs. the LPS group, $ $P < 0.01$ vs. the corresponding CNT group.

Table 3. Plasma Coagulatory Parameters After Intratracheal Instillation

Treatment	Fibrinogen	vWF	APC
Vehicle	318.3 ± 8.4	99.0 ± 5.2	5.6 ± 0.2
SWCNT	451.8 ± 50.1**	112.3 ± 6.4	4.1 ± 0.4**
MWCNT	340.4 ± 13.0	91.6 ± 4.0	5.0 ± 0.3
LPS	439.0 ± 27.3*	100.0 ± 3.5	4.6 ± 0.3**
LPS + SWCNT	626.3 ± 50.8**,##,$	106.5 ± 4.8	3.6 ± 0.2**,##
LPS + MWCNT	526.5 ± 30.4**,$	97.4 ± 7.2	4.6 ± 0.3**

* $P < 0.05$ vs. the vehicle group, ** $P < 0.01$ vs. the vehicle group, # $P < 0.05$ vs. the LPS group, ## $P < 0.01$ vs. the LPS group, $ $P < 0.01$ vs. the corresponding CNT group.

gate the circulatory levels of cytokine and chemokines, we measured the protein levels of TNF-α, IL-1β, MIP-1α, MCP-1, and KC in the sera 24 h after intratracheal instillation (Table 4). CNT or LPS challenge did not alter these levels as compared to vehicle challenge; however, these levels were greater in the LPS + CNT groups, particularly, the LPS + SWCNT group than in the vehicle group ($P < 0.01$ for IL-1β and MCP-1; $P < 0.05$ for MIP-1α and KC). The levels were further greater in LPS + SWCNT groups than in the LPS ($P < 0.01$ for IL-1β; $P < 0.05$ for MIP-1α and KC), or the CNT ($P < 0.01$ for MCP-1; $P < 0.05$ for KC) group.

Effects of CNT on Functions of AM and PBM. To assess the effects of CNT on functions of AM and PBM in terms of cytokine production, we harvested AM and PBM from naïve ICR mice, and then stimulated with LPS (10 μg/ml) or medium alone *in vitro* for 20 h in the presence or absence of CNT, and determined protein levels for IL-1β, TNF-α, and KC (Fig. 2). SWCNT exposure moderately elevated the IL-1β level in AM culture, although it did not reach statistical significance. LPS elevated all the levels by AM ($P < 0.01$ for TNF-α and KC). The levels for all cytokines by LPS + CNT- exposed AM were comparable to those by LPS-exposed AM. CNT exposure did not elevate the IL-1β level by PBM as compared to medium exposure. LPS moderately elevated all of the levels without significance. The levels were greater by LPS + CNT-exposed PBM than by LPS-exposed PBM ($P < 0.01$ for TNF-α, $P < 0.05$ for IL-1β).

Discussion

The present study has demonstrated that 1) pulmonary exposure to two types of CNT, particularly SWCNT, induces neutrophilic lung inflammation and pulmonary edema with enhanced lung expression of proinflammatory cytokines; 2) however, they do not significantly enhance LPS-elicited lung inflammation; 3) SWCNT induce systemic inflammation, and augment systemic inflammation with coagulopathy accompanied by lung inflammation induced by LPS; and 4) finally, CNT amplified LPS-induced cytokine production from PBM.

Short-term exposure to ambient PM is related to mortality and morbidity in individuals with cardiopulmonary predisposing factors (28–30). Consistent with epidemiological studies, we previously demonstrated that DEP, important constituents of $PM_{2.5}$, and carbon nanoparticles enhance lung inflammation related to LPS (6, 11, 13) and the accompanying systemic inflammation with coagulatory disturbance (12, 13) using the same protocol as that in the present study. Furthermore, we demonstrated that carbon nanoparticles, used as a type of ambient PM, deteriorate lung inflammation and subsequent systemic inflammation with coagulatory disturbance in the same model as used in the present study (13). Collectively, environment-existing particles play roles in the adverse effects on cardiopulmonary systems with predisposing factors such as lung and systemic inflammation and the resultant coagulopathy. On the other hand, industry-engineered materials have reportedly been increasing in the environment as well as combustion-derived nanoparticles (16, 17); thus, it can be imagined that exposure to these materials, especially nano-

Table 4. Circulatory Levels of Cytokines and Chemokines After Intratracheal Instillation

Treatment	TNF-α	IL-1β	MIP-1α	MCP-1	KC
Vehicle	4.4 ± 0.4	8.5 ± 0.4	20.4 ± 0.5	131.2 ± 3.0	164.7 ± 5.3
SWCNT	4.7 ± 0.7	15.7 ± 1.3	28.2 ± 4.7	134.0 ± 2.9	174.9 ± 6.3
MWCNT	4.6 ± 0.5	12.0 ± 1.4	20.8 ± 0.8	159.5 ± 8.9**	213.0 ± 28.0*
LPS	4.3 ± 0.6	10.0 ± 0.6	20.3 ± 0.6	149.0 ± 4.2*	170.1 ± 9.7
LPS + SWCNT	10.2 ± 3.7	24.0 ± 7.7**,##	29.6 ± 4.1*,#	156.6 ± 5.5**,$$	242.9 ± 39.3*,#,$
LPS + MWCNT	8.4 ± 2.3	10.5 ± 0.6	21.9 ± 0.8	148.5 ± 4.3*	218.7 ± 7.6

* $P < 0.05$ vs. the vehicle group, ** $P < 0.01$ vs. the vehicle group, # $P < 0.05$ vs. the LPS group, ## $P < 0.01$ vs. the LPS group, $ $P < 0.01$ vs. the corresponding CNT group.

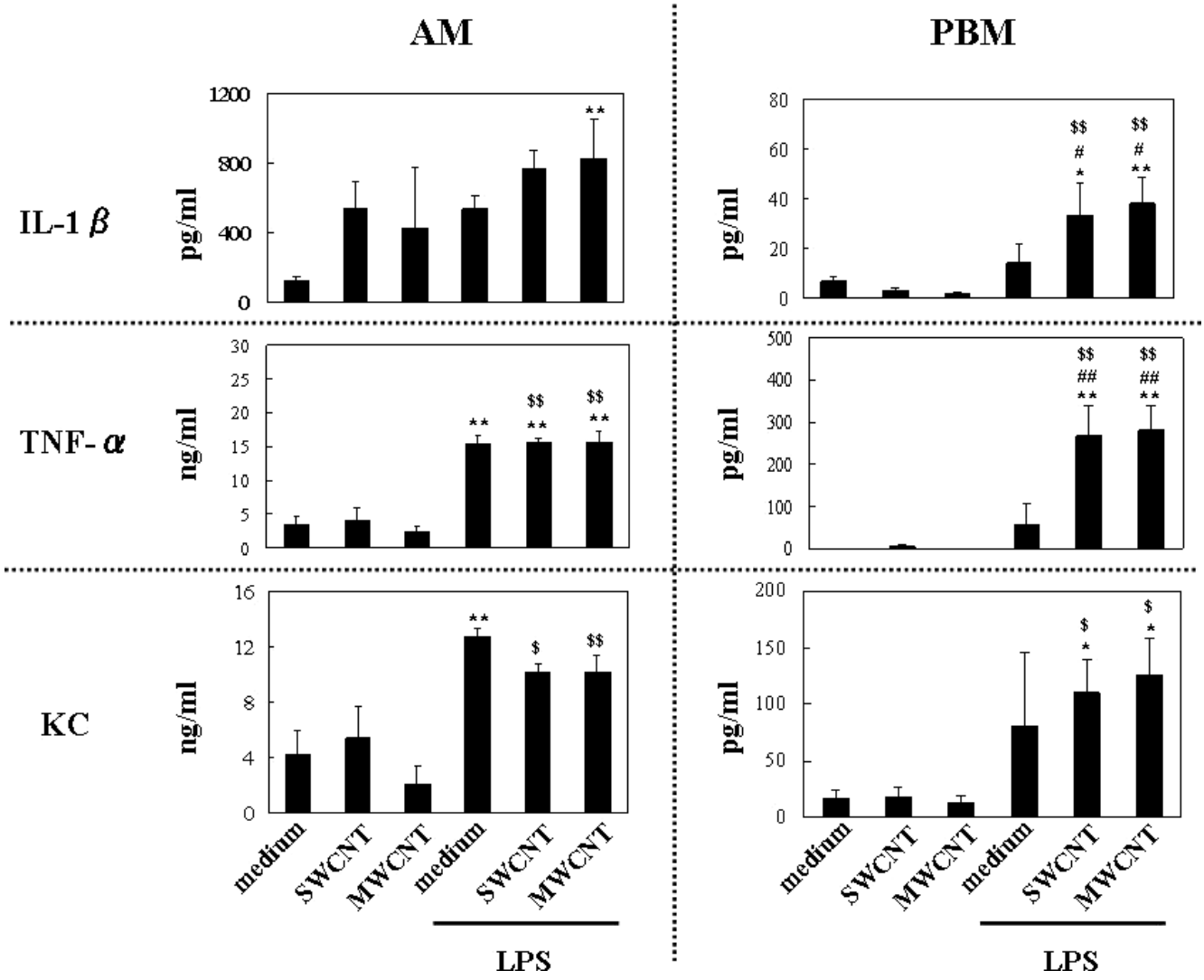

Figure 2. Cytokine production by alveolar macrophage (AM) and peripheral blood monocytes (PBM) in response to lipopolysaccharide (LPS) and/or carbon nanotubes (CNT). AM and PBM were isolated from naïve ICR mice, purified with adherence, and then stimulated *in vitro* with LPS (10 μg/ml) or with medium alone in the presence or absence of CNT for 20 h. Culture supernatants were collected for the determination of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and keratinocyte-derived chemoattractant (KC) levels by ELISA. * $P < 0.05$ vs. medium, ** $P < 0.01$ vs. medium, # $P < 0.05$ vs. LPS, ## $P < 0.01$ vs. LPS, $ $P < 0.05$ vs. CNT, $$ $P < 0.01$ vs. CNT. Results are representative of two independent experiments; $n = 3$ per condition.

sized materials, also has adverse health effects, especially inflammatory conditions. Indeed, Warheit and colleagues have demonstrated that a single intratracheal administration of 300 nm TiO_2 induces lung inflammation (31). Furthermore, a recent study has shown that a single pulmonary exposure to nano TiO_2 particles (19–21 nm) induces emphysema-like lung injury (32). Also, SWCNT instilled through the airways (pharynges) induce lung inflammation, which is characterized by fibrogenic changes with granuloma formation (33). Consistent with these previous reports, our study also showed that CNT alone induced apparent acute type of neutrophilic lung inflammation (Table 1). On the other hand, all previous studies examined the effects of (nano) materials on normal subjects/conditions in terms of toxicity, including carcinogenesis. Further, importantly, there was no report about the effects of pulmonary-exposed nanomaterials on coagulatory systems. The present results, for the first time to our knowledge, showed that exposure to two types (SWCNT and MWCNT) of manufactured CNT can facilitate both local (*i.e.,* lung) inflammation with edema and systemic inflammation with coagulatory disturbance induced by LPS.

Nano-sized particles/materials are reportedly able to affect not only the respiratory tract but also the systemic circulation (14, 34, 35), implying that they can affect the circulatory system. In accord with these studies, we previously demonstrated that carbon nanoparticles enhance systemic inflammation with coagulatory disturbance in the same model as used in the present study (13). In addition, enhancement has been greater with smaller than larger

nanoparticles (13). In this respect, a recent study by another group has shown that pulmonary exposure to MWCNT elevates circulatory levels of procoagulant microvesicular tissue factor (TF) activity, implying their enhanced thrombogenicity (36). Further, Li *et al.* have recently demonstrated that airway exposure to SWCNT induces mitochondrial damage in the aorta of wild-type mice and accelerates atherosclerosis formation in $ApoE^{-/-}$ mice (37). In the present study, LPS combined with CNT, specifically with SWCNT, significantly elevated the levels of fibrinogen, IL-1β, MCP-1, and KC in circulating blood compared to LPS alone. Additionally, the decrease in APC induced by LPS was further repressed by the combination of LPS with SWCNT. These findings suggest that CNT, especially SWCNT, can directly and/or indirectly promote systemic inflammation with coagulatory disturbance accompanied by lung inflammation, similar to nanoparticles (13). Interestingly, the degree of promotion was apparently greater in systemic inflammation with coagulopathy than in lung inflammation (Tables 1, 3, 4). It can be hypothesized that endothelial-epithelial damage induced by CNT and subsequent infiltrated effector leukocytes allow large amounts of LPS (and CNT) to pass easily into the circulation, resulting in synergistic effects on systemic inflammation and hemostasis, including coagulatory disturbance. The marked enhancing effects of SWCNT on LPS-elicited pulmonary vascular permeability might, at least in part, support this concept. Otherwise, we have previously shown by electron microscopy that intratracheally instilled carbon nanoparticles occasionally pass into the circulation through the large gap formed between alveolar epithelial cells (38). It is possible that the gap is formed as a result of shrinkage of the cytoplasm, by receiving stimulus/signals generated following nanoparticle attachment on these cells (38). Thus, it can also be proposed that SWCNT's attachment to alveolar epithelial cells enlarges the gap. Alternately, their unique shapes (fibrous with a nano-leveled cross section) may make it easier to pass through the endothelial-epithelial barrier with or without LPS and consequently to induce/augment systemic inflammation with coagulatory disturbance. In this regard, persistent reactive fibers reportedly lead to oxidative reactions (39), important reactions in vascular pathophysiology including coagulatory disorders (40). In any case, these observations may shed light on their facilitating properties of systemic inflammation with coagulopathy as well as the dynamics of (SW) CNT from the airways to the circulation, and further studies are needed to address these important issues and their mechanisms.

Innate immunity including the response against LPS initiates from macrophage/monocyte activation. Furthermore, LPS induces macrophages/monocytes to express TF, which results in intravascular fibrin deposition through activation of the coagulation protease cascade (41). To rationally explain the effects of CNT on LPS-related lung and systemic pathology, we isolated AM and PBM from the animals and cultured with LPS and/or CNT. As a result, CNT did not synergistically amplify LPS-evoked cytokine production by AM but did by PBM. Thus, the *in vitro* findings were partly linked to the *in vivo* observation that CNT did not significantly exacerbate LPS-elicited lung inflammation but rather accompanied systemic inflammation and coagulatory disturbance. Taken together, the *in vivo* effects of CNT may be in part explained by their impact on macrophage/monocyte populations. In other words, this simple *in vitro* assay system using AM and PBM might be suitable for screening the facilitating effects of toxic pollutants on this *in vivo* model and/or human inflammatory diseases (i.e. SIRS) with coagulopathy. On the other hand, however, there existed discrepant findings between *in vivo* and *in vitro* studies, especially with those on MWCNT. In fact, MWCNT did not significantly enhance systemic inflammation and coagulatory disturbance, but did LPS-induced cytokine production *in vitro*. The reason of the phenomenon remains unclarified. This may be explained by disability of the CNT to translocate from the lung into circulation, resulting in minor facilitation against systemic inflammatory responses *in vivo*. Additional studies are needed to address the issue.

In conclusion, this study demonstrated that pulmonary exposure to CNT moderately exacerbated/amplified lung inflammation, pulmonary vascular permeability, and lung expression of proinflammatory cytokines induced by LPS. The enhancing effects were more apparent with the single-walled type (SWCNT) than with multi-walled type (MWCNT) in overall trend. Furthermore, SWCNT also enhanced systemic inflammation and, more importantly, coagulatory disturbance accompanying lung inflammation. These results suggest that CNT can facilitate lung inflammation related to LPS and consequent systemic inflammation with coagulopathy. These aggravating effects of CNT might play a vital role in the adverse health effects of industrial nanomaterials on sensitive individuals with cardiovascular and respiratory diseases, including atherosclerosis and respiratory infection.

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