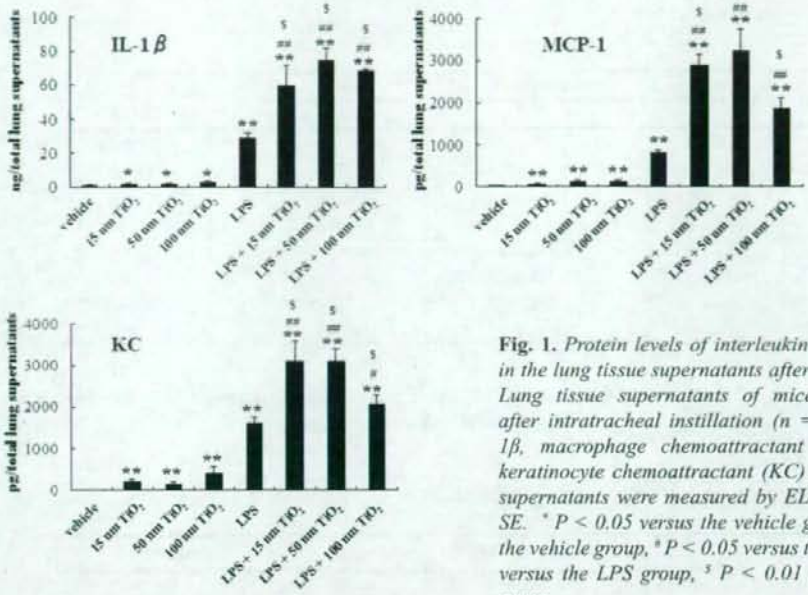

Fig. 1. *Protein levels of interleukin (IL)-1β and chemokines in the lung tissue supernatants after intratracheal challenge. Lung tissue supernatants of mice were harvested 24 h after intratracheal instillation (n = 8 in each group). IL-1β, macrophage chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC) levels in the lung tissue supernatants were measured by ELISA. Results are mean ± SE. * P < 0.05 versus the vehicle group, ** P < 0.01 versus the vehicle group, # P < 0.05 versus the LPS group, ## P < 0.01 versus the LPS group, $ P < 0.01 versus the nanomaterial group.*

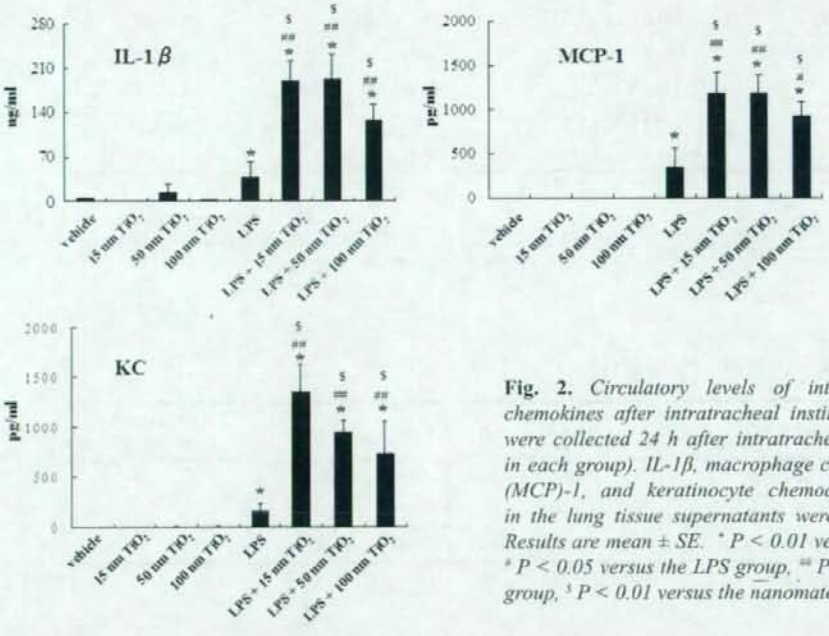

Fig. 2. *Circulatory levels of interleukin (IL)-1β and chemokines after intratracheal instillation. Blood samples were collected 24 h after intratracheal instillation (n = 10 in each group). IL-1β, macrophage chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC) levels in the lung tissue supernatants were measured by ELISA. Results are mean ± SE. * P < 0.01 versus the vehicle group, # P < 0.05 versus the LPS group, ## P < 0.01 versus the LPS group, $ P < 0.01 versus the nanomaterial group.*

Table I. *The number of total cells and neutrophils and protein concentration in bronchoalveolar lavage (BAL) fluid and lung water content after intratracheal challenge.*

Treatment	Total cells ($\times 10^6$/total BAL fluid)	Neutrophils ($\times 10^6$/total BAL fluid)	Protein (mg/ml of BAL fluid)	Lung wet weight - dry weight [mg]/body weight [g]
vehicle	70.3 ± 7.1	21.4 ± 2.9	0.32 ± 0.02	4.17 ± 0.11
15 nm TiO_2	75.9 ± 24.6	37.5 ± 3.9	0.47 ± 0.04	4.74 ± 0.05 *
50 nm TiO_2	81.6 ± 36.6	44.6 ± 7.4 *	0.50 ± 0.03	4.63 ± 0.12 *
100 nm TiO_2	127.8 ± 34.9	81.1 ± 9.7 *	0.45 ± 0.03	4.52 ± 0.08
LPS	584.4 ± 47.3 **	511.7 ± 42.2 **	0.59 ± 0.06 *	6.72 ± 0.11 **
LPS + 15 nm TiO_2	725.0 ± 116.0 **#$	647.6 ± 105.6 **#$	0.95 ± 0.07 **#$	7.69 ± 0.20 **#$
LPS + 50 nm TiO_2	711.6 ± 155.4 **$	621.1 ± 136.8 **$	1.14 ± 0.15 **##$	7.43 ± 0.21 **##$
LPS + 100 nm TiO_2	547.2 ± 46.5 **$	476.3 ± 43.1 **$	1.08 ± 0.09 **#$	7.67 ± 0.20 **##$

Results are mean ± SE. * $P < 0.05$ *versus the vehicle group,* ** $P < 0.01$ *versus the vehicle group,* # $P < 0.05$ *versus the LPS group,* ## $P < 0.01$ *versus the LPS group,* $ $P < 0.01$ *versus the nanomaterial group*

Table II. *Plasma coagulatory parameters after intratracheal instillation.*

Treatment	Fibrinogen (mg/dl)	vWF (%)	Activity of PC (%)
vehicle	359.0 ± 7.1	73.4 ± 5.9	4.6 ± 0.7
15 nm TiO_2	405.5 ± 17.1	71.9 ± 5.2	4.9 ± 0.4
50 nm TiO_2	424.0 ± 18.1	66.4 ± 7.0	4.9 ± 0.2
100 nm TiO_2	439.0 ± 17.7 *	64.9 ± 4.1	4.9 ± 0.2
LPS	654.8 ± 24.1 **	90.4 ± 7.2 **	3.5 ± 0.2 *
LPS + 15 nm TiO_2	833.0 ± 25.2 **##$	104.4 ± 3.0 **##$	2.4 ± 0.2 **$
LPS + 50 nm TiO_2	765.5 ± 42.6 **#$	105.0 ± 5.3 **##$	2.8 ± 0.3 **$
LPS + 100 nm TiO_2	745.4 ± 27.5 **#$	99.9 ± 8.0 **#$	2.7 ± 0.3 **$

Results are mean ± SE. * $P < 0.05$ *versus the vehicle group,* ** $P < 0.01$ *versus the vehicle group,* # $P < 0.05$ *versus the LPS group,* ## $P < 0.01$ *versus the LPS group,* $ $P < 0.01$ *versus the nanomaterial group*

Table III. *Number of up- or down-regulated genes.*

Treatment	2.0 <	6.0 <	10.0 <	< 0.5	< 0.16	< 0.1	inflammatory response (6.0 <)
15 nm TiO_2 vs. vehicle	961	107	40	1210	89	46	0
LPS vs. vehicle	1450	244	96	1627	89	41	22
LPS + 15 nm TiO_2 vs. vehicle	1391	221	85	1665	103	49	23

fashion (n = 4-5 in each group). ELISAs for interleukin (IL)-1β, macrophage chemoattractant protein (MCP)-1, and keratinocyte chemoattractant (KC: R&D Systems, Minneapolis, MN) in the lung tissue supernatants (n = 8 in each group) were conducted as previously described (5, 8).

Assays for circulatory levels of fibrinogen, von Willebrand factor (vWF), IL-1β, MCP-1, and KC, and activity of protein C (PC)

Citrate plasma levels of fibrinogen and vWF, and the activity of PC (n = 8-10 in each group) were determined using commercial kits (Diagnostica Stago, Roche, Tokyo, Japan) on STA Compact (Diagnostica Stago, Roche) as described previously (17). Serum samples (n = 10 in each group) were analyzed by ELISA for IL-1β, MCP-1, and KC (R & D Systems) according to the manufacturer's instructions as described above, and the values were expressed as pg/ml.

Microarray analysis

Total RNAs in the murine lungs from the vehicle, the 15 nm TiO_2, the LPS, or the LPS + 15 nm TiO_2 group were extracted with ISOGEN (Nippon Gene, Tokyo, Japan) 4 h after the intratracheal inoculation and then purified using an RNeasy mini kit (QIAGEN Pty. Ltd., Clifton, Victoria) according to the manufacturer's instructions. Total RNA was converted to cDNA with SuperScript TM choice system for cDNA synthesis (Invitrogen, Carlsbad, CA) and subsequently converted to biotinylated cRNA with an Enzo High Yield RNA Transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Microarray hybridization was performed using MouseExpression Array 430 2.0 Array (Affymetrix, Santa Clara, CA). After hybridization, the gene chips were automatically washed and stained with streptavidin-phycoerythrin by using a fluidics system. The chips were scanned with a GeneChipScanner3000. From data image files, gene transcript levels were determined using algorithms in the Microarray Analysis Suite Version 5 software (Affymetrix). This analysis was performed with four mice per chip in each group. Differences between the data from vehicle- and nanomaterial-treated mice, vehicle- and LPS-treated mice, and vehicle- and LPS + nanomaterial-treated mice were determined using GeneChipOperatingSoftware (Silicon Genetics, Redwood City, CA). Genes that were significantly up-regulated by more than 2-, 6-, and 10-fold, or down-regulated by more than 0.5-, 0.16-, and 0.1-fold in an experiment were averaged. The genes were categorized by the biological process using NetAffyx Analysis (Affymetrix; http://www.affymetrix.com/analysis/index.affx).

Statistical analysis

Data are reported as means ± SE. Differences between groups were determined using analysis of variance (ANOVA: Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA). If differences between groups were significant ($P < 0.05$) using one-way ANOVA, the Bonferroni correction was used for multiple comparison.

RESULTS

Effects of nanomaterials on airway inflammation and pulmonary vascular permeability

To estimate the magnitude of airway inflammation, we examined the cellular profile of BAL fluid 24 h after the intratracheal instillation (Table I). TiO_2 alone increased the number of total cells and neutrophils as compared with vehicle ($P < 0.05$ for the number of neutrophils with 50 nm or 100 nm TiO_2). LPS exposure significantly increased the numbers as compared with vehicle exposure ($P < 0.01$). The numbers were even greater in the LPS + 15 nm TiO_2 or LPS + 50 nm TiO_2 group than in the LPS group ($P < 0.05$ for LPS + 15 nm TiO_2 group) or the corresponding nanomaterial groups ($P < 0.01$). Next, to estimate pulmonary vascular permeability, we examined the protein levels in BAL fluid and the lung water content 24 h after the intratracheal instillation. Pulmonary exposure to TiO_2 elevated the protein levels of BAL fluid and the lung water content as compared to exposure to vehicle ($P < 0.05$ for the lung water content with 15 nm or 50 nm TiO_2). These values were significantly greater in the LPS group ($P < 0.05$ for the protein level of BAL fluid, $P < 0.01$ for the lung water content). These values were even greater in the LPS + TiO_2 groups than in the LPS group ($P < 0.01$, except in the case of the protein level of BAL fluid in the LPS + 15 nm TiO_2 group; $P < 0.05$) or the corresponding nanomaterial groups ($P < 0.01$).

Effects of nanomaterials on histological changes in the lung

To determine the effects of nanomaterials on lung histology, we evaluated lung specimens stained with hematoxylin and eosin 24 h after the intratracheal instillation. No pathological change was seen in the lung obtained from the vehicle groups. Infiltration of neutrophils was seen slightly in the lungs from the nanomaterial groups and moderately seen in those from the LPS groups. Combined treatment with nanomaterials and LPS markedly enhanced

leukocyte (mainly neutrophil) sequestration into the lung parenchyma as compared with LPS treatment alone, especially with smaller nanomaterials with overall trend. Furthermore, we performed morphometric analysis to quantitate the number of PMNs in the lung. TiO_2 alone significantly increased the number as compared with vehicle (the number ± SE: vehicle, 0.9 ± 0.3; 15 nm TiO_2, 4.4 ± 1.0; 50 nm TiO_2, 3.7 ± 0.8; 100 nm TiO_2, 3.4 ± 0.1). LPS challenge significantly increased the numbers as compared with vehicle exposure (46.8 ± 9.7, $P < 0.01$). The numbers were further increased in the LPS + 15 nm TiO_2 (193.2 ± 29.1), the LPS + 50 nm TiO_2 (124.7 ± 20.5), and the LPS + 100 nm TiO_2 (103.8 ± 14.9) group compared to the LPS group ($P < 0.01$) or the corresponding nanomaterial group ($P < 0.01$).

Effects of nanomaterials on the expression of proinflammatory cytokine and chemokines in the lung

To investigate the effects of nanomaterials on the local cytokine and chemokine expression related to LPS, we next measured the protein levels of IL-1β, MCP-1, and KC in the lung tissue supernatants 24 h after the intratracheal instillation (Fig. 1). Pulmonary exposure to TiO_2 alone significantly elevated these levels as compared to exposure to vehicle ($P < 0.05$ for the level of IL-1β; $P < 0.01$ for the level of MCP-1 or KC). LPS challenge significantly elevated the levels of all of the proteins as compared with vehicle challenge ($P < 0.01$). The levels were even greater in the LPS + TiO_2 groups than in the LPS ($P < 0.01$; except for vs. LPS + 100 nm TiO_2 group for the KC level; $P < 0.05$) or the corresponding nanomaterial ($P < 0.01$) groups.

Effects of nanomaterials on coagulatory changes

To investigate the impact of pulmonary exposure to nanomaterials on the coagulatory system, we next analyzed coagulatory parameters 24 h after the intratracheal challenge (Table II). The fibrinogen level was elevated by TiO_2 exposure as compared to vehicle exposure ($P < 0.05$ vs. 100 nm TiO_2). LPS challenge significantly elevated the values as compared to vehicle challenge ($P < 0.01$). The values were further greater in the LPS + nanomaterial groups than in the LPS group ($P < 0.05$ for the LPS + 50 nm or the LPS + 100 nm nanomaterial group; $P < 0.01$ for the LPS + 15 nm nanomaterial group) or the corresponding nanomaterials groups ($P < 0.01$). As compared to vehicle challenge, LPS challenge caused a significant increase in the level of vWF ($P < 0.01$). The level was even greater in the LPS + TiO_2 groups than in the LPS group ($P < 0.05$ for the LPS + 100 nm TiO_2 group; $P < 0.01$ for the LPS + 15 nm TiO_2 or the LPS + 50 nm TiO_2 group) or the corresponding nanomaterial group ($P < 0.01$). LPS significantly decreased the activity of PC as compared with vehicle ($P < 0.05$). The activity was further decreased in the LPS + TiO_2 groups than in the LPS group (N. S.) or the corresponding latex groups ($P < 0.01$).

Effects of nanomaterials on circulatory levels of proinflammatory cytokine and chemokines

To investigate the circulatory levels of cytokine and chemokines related to LPS, we measured the protein levels of IL-1β, MCP-1, and KC in the sera 24 h after the intratracheal instillation (Fig. 2). LPS challenge significantly elevated these levels ($P < 0.01$) as compared to vehicle challenge. The levels were further increased in the LPS + TiO_2 groups compared to the LPS ($P < 0.01$; except for vs. LPS + 100 nm TiO_2 group; $P < 0.05$), or the nanomaterial ($P < 0.01$) group.

Effects of nanomaterials on gene expression patterns in the lung

Finally, we performed cDNA microarray analysis to determine the effects of nanomaterials on the global gene expression profile in LPS-related lung inflammation (Table III). mRNA was prepared from the murine lung 4 h after the intratracheal instillation in each group. The number of up- or down- regulated genes vs. those in the vehicle group was greater in the LPS group or the LPS + 15 nm TiO_2 group than in the 15 nm TiO_2 group. However, the number was comparable between the LPS and the LPS + 15 nm TiO_2 groups.

DISCUSSION

This study demonstrates that TiO_2 nanomaterials instilled intratracheally enhance neutrophilic lung inflammation with pulmonary vascular permeability

related to LPS. This is concomitant with the increased local (lung) expressions of proinflammatory cytokine such as IL-1β, and chemokines such as MCP-1 and KC. In addition, combined challenge with LPS and nanomaterials further increases circulatory levels of fibrinogen, vWF, IL-1β, MCP-1, and KC, as compared with challenge with LPS alone. The enhancing effects of nanomaterials on the pathology tend to be overall greater with the smaller nanomaterials than with larger ones.

Short term exposure to ambient PM is related to mortality and morbidity in individuals with cardiopulmonary predisposing factors (18-20). Consistent with the epidemiological studies, we previously demonstrated that DEP, important constituents in PM, enhance lung inflammation related to LPS (5) *in vivo*. We also showed that residual carbonaceous cores of DEP after extraction with dichloromethane (washed DEP) worsens lung inflammation related to LPS (6) and the accompanying coagulatory disturbance (7) using a similar protocol to that in the present study. Furthermore, we demonstrated that carbon nanoparticles, used as a type of ambient PM, cause deterioration of lung inflammation in the same model as used in the present study (8). In that study, smaller (14 nm) nanoparticles more markedly aggravated the features than larger (56 nm) ones (8). Collectively, environment-existing particles play roles in the adverse effects of ambient PM on cardiopulmonary systems with predisposing factors such as lung inflammation. Moreover, it can be imagined that exposure to materials, especially nano-sized materials generated from industrial nano-technology, also induces adverse health effects. Indeed, Warheit and colleagues demonstrated that a single intratracheal administration of 300-nm-sized TiO_2 induces lung inflammation (12). Furthermore, a recent study showed that a single pulmonary exposure to nano TiO_2 particles (19-21 nm) induces emphysema-like lung injury (13). Also, single-walled carbon nanotubes (1 ~ 4 nm) instilled through the airways (pharynges) induce lung inflammation, which is characterized by fibrogenic changes with granuloma formation (14). However, all these studies examined the effects of (nano) materials on normal subjects/conditions. Thus, the effects of exposure to nanomaterials in individuals with predisposing conditions remain to be explored. Furthermore, investigations focusing on their size effects have never been reported. The present results have, for the first time as far as we know, shown that exposure to manufactured nanomaterials can exaggerate both local (lung) inflammation and systemic inflammation with coagulatory disturbance related to LPS, and that the effects are greater with the smaller materials than with the larger ones with overall trend.

The pathogenesis of acute lung inflammation reportedly involves amplified lung expression of proinflammatory cytokines such as IL-1β and tumor necrosis factor (TNF)-α and chemokines such as IL-8 and MCP-1 (21-23). In our previous studies, we confirmed that the lung expression of proinflammatory cytokine and chemokines including IL-1β, MCP-1, and KC in the lung 24 h after the intratracheal administration of LPS, DEP, washed DEP, or carbon nanoparticles is concomitant with the aggravated lung injuries (5-6, 8, 24). In the present study, likewise, the lung expression of these molecules paralleled the lung inflammation with overall trend (Table II). Thus, the exaggerating effects of nanomaterials on lung inflammation appear to be mediated, at least in part, through the enhanced lung expression of IL-1β, MCP-1, and KC (a murine homologue of IL-8).

Nano-sized particles or materials are reportedly able to penetrate deeply into the respiratory tract and can even pass the lung to reach the systemic circulation (25-26), implying that they can affect the circulatory system. We previously demonstrated that carbon nanoparticles enhance systemic inflammation with coagulatory disturbance in the same model as used in the present study (8). In the present study, LPS combined with nanomaterials, specifically with those with a diameter of less than 50 nm, significantly elevated the levels of fibrinogen, IL-1β, MCP-1, and KC compared to LPS alone. Additionally, the elevation of the level of vWF induced by LPS was further increased by the combination of LPS with nanomaterials. These findings suggest that nanomaterials, in particular smaller ones, can facilitate systemic inflammation with coagulatory disturbance accompanied by lung inflammation, like combustion-derived nanoparticles (8). Interestingly, on the other hand, exposure to nanomaterials alone did not induce

significant production/release of fibrinogen or vWF in the current study. It can be hypothesized that endothelial-epithelial damage induced by LPS and subsequent infiltrated effector leukocytes allow large amounts of smaller nanomaterials to pass easily into the circulation, resulting in synergistic effects on systemic inflammation and hemostasis, including coagulatory disturbance. The current comparative results, in which nanomaterials alone induced significant expression of proinflammatory cytokines (IL-1β, MCP-1, and KC) in the lung homogenates, whereas they did not in the serum, favor this concept. In addition, the marked enhancing effects of nanomaterials on the LPS-elicited pulmonary vascular permeability of both water and protein might, at least in part, support this notion. A previous *in vitro* study (27) and our recent *ex vivo* study (Inoue et al., manuscript in submission) demonstrated that nanomaterials (particles) can activate monocytes to produce/release proinflammatory cytokines. Therefore, it is also possible that intratracheally instilled nanomaterials, especially smaller ones, enter the circulation, and activate monocytes in the circulation, resulting in an increase in susceptibility to LPS-elicited systemic inflammation and subsequent coagulatory disturbance.

Compared to the previous experiments using carbon nanoparticles (4 mg/kg) (8), however, the nanomaterials needed a two-fold higher dose (8 mg/kg) to achieve significant enhancing effects on the lung inflammation model. This difference may be in part explained by the ability to generate oxidative stress. Oxidative stress, caused by ambient particles or nanomaterials deposited in the lung, is believed to be an important factor in causing proinflammatory and noxious effects (28). However, as compared to carboneous particles, TiO_2 generate relatively less oxidative stress (29-30). Supporting this notion, DNA microarray analyses in the present study also demonstrated that the number of up-regulated genes related to "oxidative stress" was notably smaller than that related to "inflammatory response" (Table III, and data not shown). Alternatively, there may be other differences in surface characteristic that led to this different extent of proinflammatory response.

Finally, in the current study, we did not examine the effects of TiO_2 as soluble metal salts on this model. Since the effects of TiO_2 salts, especially on DNA, could have been correlated with those of these nanomaterials, future investigations should address the correlation.

In conclusion, this study demonstrated that pulmonary exposure to industry-related nanomaterials enhanced lung inflammation and pulmonary vascular permeability induced by LPS. The enhancement was mediated through the increased local expression of proinflammatory cytokines and chemokines. The effects were more prominent with smaller nanomaterials than with larger ones in overall trend. Smaller nanomaterials also enhanced systemic inflammation and coagulatory disturbance accompanying the lung inflammation. These aggravating effects of nanomaterials might play a vital role in the adverse health effects of industrial materials on the sensitive populations who have cardiovascular and respiratory diseases.

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