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Effects of nano particles on cytokine expression in murine lung in the absence or presence of allergen

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Abstract Particulate matter (PM) can exacerbate allergic airway diseases. Health effects of PM with a diameter of less than 100 nm, called nano particles, have been focused. We have recently demonstrated that carbon nano particles (14, 56 nm) exaggerate allergic airway inflammation in mice. In the present study, we investigated the effects of repeated pulmonary exposure to carbon nano particles on the expression of a variety of cytokines in the absence or presence of allergen in mice. ICR mice were divided into six experimental groups. Vehicle, two sizes of carbon nano particles, ovalbumin (OVA), and OVA + nano particles were administered intratracheally. Nano particles increased the lung protein levels of thymus and activation-regulated chemokine (TARC), macrophage inflammatory protein (MIP)−1α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the absence or presence of allergen. The enhancement was more prominent with 14 nm of nano particles than with 56 nm of nano particles in overall trend. 14 nm nano particle exposure significantly enhanced the lung expressions of interleukin (IL)-2 and IL-10 in the presence of allergen as compared with allergen exposure. These results suggest that pulmonary exposure to nano particles can induce the lung expression of TARC, MIP-1α, GM-CSF in the absence of allergen and can enhance that of TARC, MIP-1α, GM-CSF, IL-2, and IL-10 in the presence of allergen. The enhancing effects are more prominent with smaller particles.

Keywords Nano particle · Allergen · Cytokine

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Introduction

Previous epidemiological studies have indicated that exposure to ambient particulate matter (PM) is linked to increases in mortality and morbidity related to respiratory diseases (Abbey et al. 1999; Cohen and Pope 1995). The concentration of PM with a mass median aerodynamic diameter (a density-dependent unit of measure used to describe the diameter of the particle) < or 10 μm (PM10) is related to daily hospital admissions for several respiratory diseases (Dockery et al. 1993). Recent data have shown that PM with a mass median aerodynamic diameter < or 2.5 μm (PM2.5) are more closely associated with both acute and chronic respiratory effects and subsequent mortality than PM10 (Peters et al. 1997).

Recently, nano particles, particles less than 0.1 μm in mass median aerodynamic diameter, have been implicated to affect cardiopulmonary systems (Peters et al. 1997; Utell and Frampton 2000). Indeed, two in vivo studies have demonstrated that nano particle exposure results in prominent airway inflammation as compared with larger particle exposure (Ferin et al. 1992; Li et al. 1999). Since nano particles can penetrate deeply into the respiratory tract and have a larger surface area than the particles with larger size, they can cause a greater inflammatory response (MacNee and Donaldson 2000; Nemmar et al. 2001).

Bronchial asthma has been recognized as chronic airway inflammation that is characterized by eosinophils and lymphocytes. Various particles including carbon black (CB) can enhance allergic sensitization (Lambert et al. 1999, 2000; Maejima et al. 1997). CB has been demonstrated to enhance proliferation of antibody forming cells and both IgE and IgG levels (Lovik et al. 1997; van Zijverden et al. 2000). Ultrafine particles (PM

and CB) reportedly exaggerate allergic airway inflammation in vivo (mice and rats) (Al-Humadi et al. 2002; Last et al. 2004). However, no study had elucidated the size effects of nano particles on subjects with respiratory inflammation. Recently, we have reported that carbon nano particles aggravate allergic airway inflammation in vivo (Inoue et al. 2005). Furthermore, nano particles exaggerate goblet cell hyperplasia elicited by allergen. In overall trends, the enhancing effects have been more prominent with 14 nm nano particles than with 56 nm nano particles, which are concomitant with the expression of Th2 cytokines including interleukin (IL)-5 and IL-13. In the study, however, repeated exposure to nano particles has induced airway inflammation also in the absence of allergen. Thus, there should be the other contributing inflammatory cytokines in the airway inflammation caused by nano particles.

The aim of the present study was to elucidate the effects of two sizes of carbon nano particles (14 nm or 56 nm) on the lung expression of a variety of cytokines and chemokines in the absence or presence of allergen.

Materials and methods

Animals

Male ICR mice 6–7 week of age and weighing 29–33 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. Mice were housed in an animal facility that was maintained at 24–26°C with 55–75% humidity and a 12 h light/dark cycle.

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 (Nakalai Tesque, Kyoto, Japan) once a week for 6 weeks. The ovalbumin (OVA) group received 1 μg of OVA (Sigma Chemical, St. Louis, MO) dissolved in the same vehicle every 2 week for 6 weeks (four times in total administration). The nano particle groups received 50 μg of each size of carbon nano particles (14 nm: PrinteX 90 or 56 nm: PrinteX 25, degussa, Dusseldorf, Germany) suspended in the same vehicle every week for 6 weeks (seven times in total administration). The OVA + nano particle groups received the combined treatment in the same protocol as the OVA and the nano particle groups, respectively. The surface area of the 14 nm nano particles was 300 m^2/g and that of 56 nm nano particles was 45 m^2/g. The size of each particle was quantified by JEM-2010 transmission electron microscope (TEM; JEOL, Tokyo, Japan). Nano particles were autoclaved at 250°C for 2 h before use. The suspension was sonicated for 3 min using an Ultrasonic disrupter (UD-201; Tomy Seiko, Tokyo, Japan). In each group, vehicle, OVA, nano particles, or OVA + nano particles was 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). The animals were studied 24 h after the last intratracheal administration. 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.

Quantitation of cytokine and chemokine protein levels in the lung tissue supernatants

The animals were exsanguinated and the lungs were subsequently homogenized with 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM ethylenediaminetetraacetic acid (Sigma, St Louis MO), 0.1 mM phenylmethanesulphonyl fluoride (Nacalai Tesque, Kyoto, Japan), 1 μM pepstatin A (Peptide Institute, Osaka, Japan) and 2 μM leupeptin (Peptide Institute) as described previously (Takano et al. 1997). The homogenates were then centrifuged at 105,000 *g* for 1 h. The supernatants were stored at −80°C. Enzyme-linked immunosobent assays (ELISA) for thymus and activation-regulated chemokine (TARC), macrophage inflammatory protein (MIP)−1α, IL-10 (R&D systems, Minneapolis, MN), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-2 (Endogen, Cambridge, MA) in the lung tissue supernatants were conducted using matching antibody pairs according to the manufacture's instruction. The second antibodies were conjugated to horseradish peroxidase. Subtractive reading of 550 nm from the reading at 450 nm were converted to pg/ml using values obtained from standard curves generated with varying concentrations of recombinant TARC, MIP-1α, IL-10, GM-CSF, and IL-2, with limits of detection of 5, 1.5, 4, 5, and 3 pg/ml, respectively.

Statistical analysis

Data were reported as mean ± SEM. Differences in cytokine protein levels between groups were determined using analysis of variance (Stat view version 4.0; Abacus Concepts, Inc., Berkeley, CA). If differences between groups were significant ($P < 0.05$), Fisher's protected least significant difference test was used to distinguish between pairs of groups.

Results

Effects of nano particles on the lung expression of TARC, MIP-1α, and GM-CSF in the absence or presence of allergen

To evaluate the effect of nano particles on the lung expression of TARC, MIP-1α, and GM-CSF in the

absence or presence of allergen, we investigated the lung expression in the six experimental groups (Table 1).

Nano particles significantly elevated the level of TARC in the absence of allergen as compared with vehicle ($P < 0.01$). Allergen also significantly elevated the level as compared with vehicle ($P < 0.05$). The level was significantly greater in the OVA + nano particle groups than in the OVA ($P < 0.01$) or nano particle groups ($P < 0.05$ for 56 nm nano particle, $P < 0.01$ for 14 nm nano particle). Nano particles significantly elevated the level of MIP-1α in the absence of allergen as compared with vehicle ($P < 0.01$). Allergen did not change the level as compared to vehicle. The level was significantly greater in the OVA + nano particle groups than in the OVA group ($P < 0.05$ for 56 nm nano particle, $P < 0.01$ for 14 nm nano particle). The level was significantly greater in the OVA + 14 nm nano particle group than in the 14 nm nano particle group ($P < 0.05$). Nano particles significantly elevated the level of GM-CSF in the absence of allergen as compared with vehicle ($P < 0.01$). Allergen did not affect the level as compared to vehicle. The level was significantly greater in the OVA + 14 nm nano particle group than in the OVA group ($P < 0.01$). There were no significant differences between the nano particle groups and the OVA + nano particle groups.

Effects of nano particles on the lung expression of IL-2 and IL-10 in the presence or the absence of allergen

To investigate the lung expression of IL-2 and IL-10, we measured protein levels of these cytokines in the lung tissue supernatants (Table 2). IL-2 level was significantly greater in the OVA + 14 nm nano particle group than in the vehicle, OVA, or 14 nm nano particle group ($P < 0.01$). On the other hand, IL-10 level was significantly greater in the OVA + 14 nm nano particle group than in the OVA group ($P < 0.05$).

Discussion

In the present study, repeated pulmonary exposure to carbon nano particles induced the lung expression of TARC, GM-CSF, and MIP-1α in mice. Also, nano particles enhanced the lung expression of TARC, GM-CSF, MIP-1α, IL-2, and IL-10 in the presence of allergen as compared with allergen exposure alone. In overall trends, the enhancing effects in the presence or the absence of allergen were more prominent with 14 nm nano particles than with 56 nm nano particles.

DEP exacerbate allergic airway inflammation (Takano et al. 1997). Elementary carbon, which is mainly involved in the nuclei of DEP, can enhance allergic sensitization (Maejima et al. 1997). CB exacerbates airway inflammation related to allergen in rats (Al-Humadi et al. 2002). Also, ambient particles with a diameter of less than 2.5 μm partially exacerbate airway inflammation related to allergen (Last et al. 2004). Our prior study has demonstrated that two sizes of carbon nano particles can exaggerate allergic airway inflammation, which has been evidenced by bronchoalveolar lavage (BAL) cellularity and histological findings in vivo (Inoue et al. 2005). The enhancing effects have been concomitant with the increased lung expression of IL-5, eotaxin, IL-13, regulated on activation and normal T cells expressed and secreted (RANTES), macrophage chemoattractant protein (MCP) -1, and IL-6, which are reported to be important in allergic asthma. Furthermore, particles with a diameter of 14 nm have exhibited adjuvant activity for total IgE and allergen-specific production of IgG and IgE. In the study, however, repeated exposure to nano particles has induced airway inflammation also in the absence of allergen. Therefore, there should be other contributing inflammatory proteins in the airway inflammation caused by nano particles.

Thymus and activation-regulated chemokine is reportedly produced by antigen presenting cells, mainly by dendritic cells, and induces selective migration of CCR4-expressing Th2 cells (Imai et al. 1996, 1997; Peh

Table 1 Protein levels (pg/total lung tissue supernatants) of thymus and activation-regulated chemokine (TARC), macrophage inflammatory protein (MIP)-1α and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the lung tissue supernatants

Group	TARC	MIP-1$_\alpha$	GM-CSF
Vehicle	28.8 ± 2.9	8.9 ± 0.7	12.4 ± 0.5
14 nm	100.3 ± 13.3**	207.5 ± 10.8**	27.3 ± 1.4**
56 nm	97.6 ± 12.3**	113.5 ± 12.2**	23.4 ± 0.7**
OVA	56.2 ± 16.6*	11.6 ± 2.1	12.8 ± 0.3
OVA + 14 nm	183.2 ± 20.4** $^{\#\#}$ $^{\$\$}$	365.6 ± 36.5**$^{\#\#}$ $^{\$}$	25.2 ± 1.1** $^{\#\#}$
OVA + 56 nm	149.3 ± 23.5** $^{\#\#}$ $^{\$}$	89.5 ± 23.2* $^{\#}$	18.0 ± 1.1*

Six groups were intratracheally inoculated with vehicle, nano particles, OVA, or the combination of OVA and nano particles for 6 weeks. Lungs were removed 24 h after the last intratracheal administration. Protein levels in the lung tissue supernatants were analysed using ELISA. Results are shown as mean ± SEM

$^{*}P < 0.05$ versus vehicle

$^{**}P < 0.01$ versus vehicle

$^{\#}P < 0.05$ versus OVA

$^{\#\#}P < 0.01$ versus OVA

$^{\$}P < 0.05$ versus nano particles

$^{\$\$}P < 0.01$ versus nano particles

Table 2 Protein levels (pg/total lung tissue supernatants) of interleukin (IL)-2 and IL-10 in the lung tissue supernatants

Group	IL-2	IL-10
Vehicle	50.5 ± 4.6	6.7 ± 1.3
14 nm	41.7 ± 2.9	7.2 ± 1.4
56 nm	44.1 ± 5.0	6.9 ± 1.6
OVA	52.7 ± 3.8	5.3 ± 1.3
OVA + 14 nm	86.5 ± 8.6$^{*\ \#\#\ \$}$	9.4 ± 1.4$^{\#}$
OVA + 56 nm	51.1 ± 5.3	5.1 ± 1.0

Six groups were intratracheally inoculated with vehicle, nano particles, OVA, or the combination of OVA and nano particles for 6 weeks. Lungs were removed 24 h after the last intratracheal administration. Protein levels in the lung tissue supernatants were analysed using ELISA. Results are shown as mean ± SEM

$^{*}P < 0.01$ versus vehicle

$^{\#}P < 0.05$ versus OVA

$^{\#\#}P < 0.01$ versus OVA

$^{\$}P < 0.01$ versus nano particles

et al. 2001; Zlotnik and Yoshie 2000). Neutralization of TARC in vivo results in the inhibition of the Th2-dominant response (Yoneyama et al. 1998). TARC is reportedly highly expressed in lesions of atopic disorders such as dermatitis (Kakinuma et al. 2001) and asthma (Berin et al. 2001; Panina-Bordignon et al. 2001; Sekiya et al. 2000). MIP-1α is produced by many types of cells including macrophages, dendritic cells, and lymphocytes (Maurer and von Stebut 2004), and have been shown to possess chemotactic activity for inflammatory and immune effector cells including neutrophils and mononuclear cells (Driscoll 1994). Furthermore, significant elevations of the chemokine in BAL fluid have been reportedly shown in allergic patients (Katoh et al. 2000). GM-CSF is produced by stromal cells derived from bone marrow and reportedly induces myeloid stem cells to proliferate and differentiate neutrophils, eosinophils, and monocytes (Gasson 1991). GM-CSF is also reportedly a key contributor to several inflammatory diseases and is a potent inhibitor of neutrophil and eosinophil apoptosis (Coxon et al. 1999; Tai et al. 1991). Furthermore, it has been shown that GM-CSF is produced by pulmonary granulocytes from patients with inflammatory respiratory diseases (Dibbert et al. 1999), especially by eosinophils in asthmatics (Broide et al. 1992). In the present study, repeated exposure to nano particles induced lung expressions of TARC, MIP-1α, and GM-CSF. The effects of nano particles including an increase in inflammatory cell influx into the airways in our prior study might be explained, at least partly, by the induction of these proteins, since our previous report has shown that exposure to nano particles alone also induce moderate airway inflammation on the same protocol as the present study. Alternatively, nano particles may serve several types of cells including macrophages, dendritic cells, and lymphocytes in the respiratory system in producing/secreting these cytokine and chemokines and, consequent proliferation, differentiation, and recruitment of effector cells from the circulation to the lung. Also, repeated exposure to nano particles may induce Th2-shift response in the airways. Furthermore, the levels were also greater in the OVA + nano particle groups than in the OVA group. Thus, the aggravating effects of nano particles on allergic airway inflammation might be mediated, at least in part, via the enhanced lung expression of these cytokine and chemokines.

Interleukin (IL)-2 is produced primarily by activated CD4+ cells and is a potent growth factor for T cells (Nelson 2004; Smith 1988). Also, we (Takano et al. 1997) and another group (Renzi et al. 1992) have previously shown that IL-2 is important for the modulation of allergic airway inflammation with allergen-specific Ig production in vivo. In the recent study, we have demonstrated that nano particles, especially 14 nm nano particles, aggravate allergic airway inflammation. Further, the airway inflammation has been concomitant with the enhanced Igs production. In the present study, lung expression of IL-2 was significantly greater in the OVA + 14 nm nano particle group than in the vehicle, OVA, or 14 nm nano particle group. Thus, IL-2 may contribute, at least partly, to the deteriorated allergic airway inflammation and Ig response induced by 14 nm nano particle. Also, this finding suggests that CD4+ may be target cells for the enhancing effects of 14 nm nano particles on the present allergic model.

In overall trends, the enhancing effects of nano particles on local expressions of cytokines and chemokines in the presence or absence of allergen were more prominent with 14 nm nano particles than with 56 nm nano particles. The differences in the enhanced expression of the proteins between the two sizes of nano particles may contribute, at least partly, to those in the magnitude of allergic airway inflammation in our previous study (Inoue et al. 2005). However, in our previous and present studies, we have excised the whole lung without any discrimination of alveolar cells (macrophages) or other cells. Therefore, we could not address which cells produce cytokines and chemokines related to allergen or which cells and/or inflammatory and immune systems are affected by these molecules in the present study. Future studies dividing cell population warrant useful findings.

In the real world, we inhale nano particles and allergen in ambient air, not particle suspension nor aliquot of allergen. The dose of nano particles injected in the present study can be estimated to be less than a hundred-fold than that we inhale in daily life. Further, real PM including DEP are complex mixture of carbon, metals, and organics, which are different from CB used in the present study. Thus, future inhalation studies are needed to elucidate whether daily exposure to nano particles with or without other compounds including organic chemicals than elementary carbon combined with occasional exposure of aerosol allergen lead to the same results as the present study.

In conclusion, the present study has shown evidence that pulmonary exposure to carbon nano particles can induce lung expression of TARC, GM-CSF, and MIP-

1α in the absence of allergen in mice. Nano particles can enhance lung expressions of TARC, GM-CSF, MIP-1α, IL-2, and IL-10 in the presence of allergen as compared with allergen exposure alone. The enhanced expression of these inflammatory proteins should play an important role in the airway inflammation related to nano particles in the absence or presence of allergen. Furthermore, the enhancing effect is larger with the smaller particles.

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