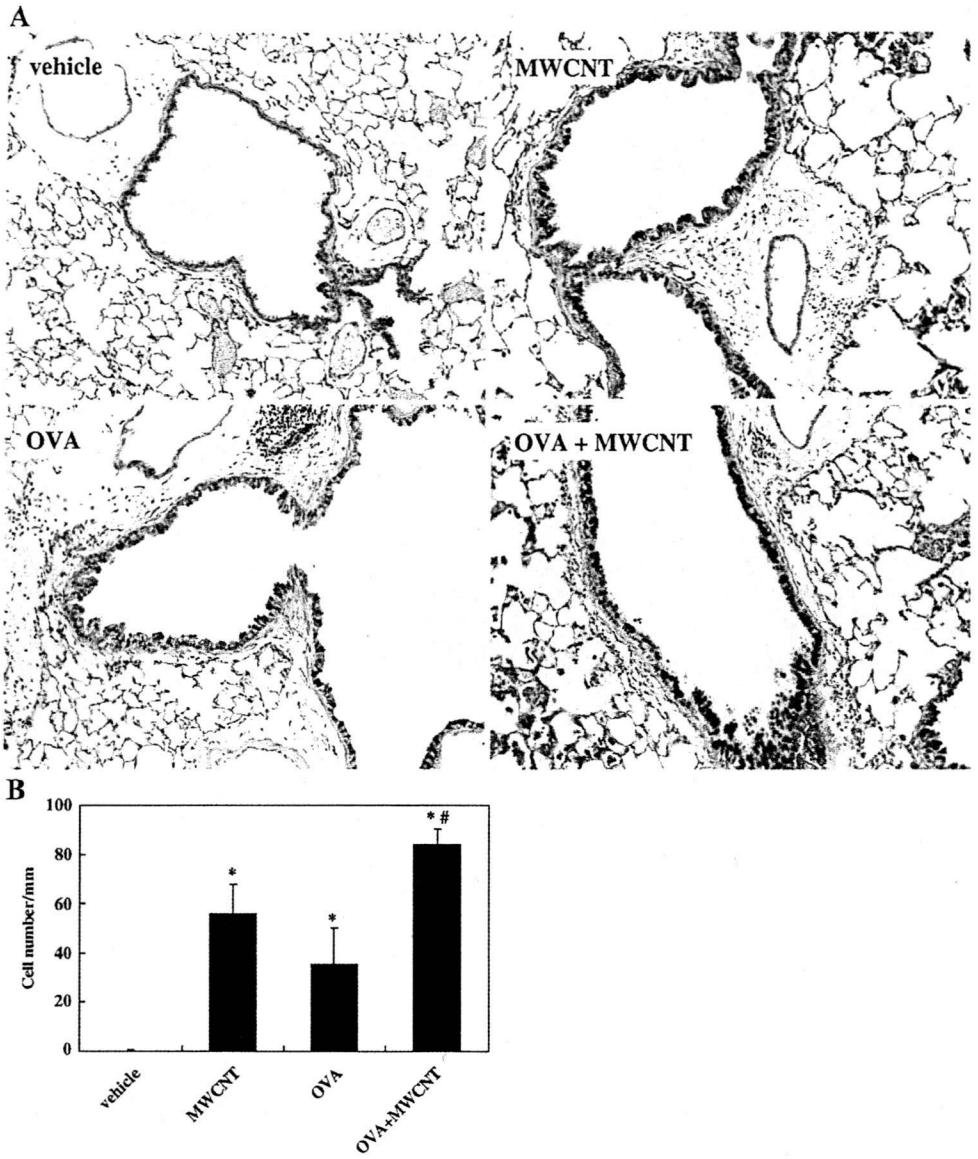

Fig. 4. Representative histological findings of the periodic acid-Schiff (PAS)-stained lungs obtained from the vehicle, MWCNT (50 μg/treatment), OVA, or OVA + MWCNT group (A), and semi-quantitative analysis of the number of goblet cells in the lung (B; $n = 4$–5 in each group). Animals received intratracheal instillation of vehicle, MWCNT, OVA, or OVA + MWCN over a period of 6 wk. Lungs were removed and fixed 24 h after the last intratracheal administration. Original magnification × 200. The number of goblet cells in the bronchial epithelium was expressed as the number of PAS-positive cells per millimeter of basement membrane. Values are the mean ± SE in each group. $^{*}P<0.01$ vs. vehicle group, $^{\#}P<0.05$ vs. OVA group.

6) on the cells was analyzed on the maturation/activation of BMDC. The percentage of MHC Class II^+ cells (Fig. 6A) and $CD86^+$ cells (Fig. 6B) was significantly increased by the addition of 0.1 and 1 μg/ml of MWCNT. Also, the percentage of MHC Class II^+CD86^+ cells (Fig. 6C) was significantly increased by the addition of 0.1 μg/ml of MWCNT. The expression (or co-expression) of other

Table 2
Protein levels of cytokines in lung tissue supernatants after the final intratracheal challenge.

Treatment	IL-4	IL-5	IL-13	IFN-γ	IL-18	IL-33
	(pg/total lung tissue supernatants)					(ng/Total lung tissue supernatants)
Vehicle	14.9 ± 2.59	84.3 ± 0.64	2.09 ± 0.85	1724.0 ± 125.0	1167.8 ± 101.5	8.0 ± 1.4
MWCNT	17.9 ± 3.91	27.1 ± 1.8**	74.1 ± 9.58**	4295.6 ± 264.9**	3716.4 ± 335.1**	264.8 ± 18.7**
OVA	32.2 ± 4.95*	196.0 ± 54.6**	235.8 ± 56.7**	2028.4 ± 128.5	922.8 ± 70.2	35.1 ± 6.2**
OVA + MWCNT	96.8 ± 12.3**#$	385.9 ± 58.9**$	343.8 ± 61.2**$	3628.6 ± 124.9**#	2972.1 ± 399.8**#	275.6 ± 26.6**#

Results are expressed as the mean ± SE ($n = 8$ in each group). $^{*}P<0.05$ vs. vehicle group, $^{**}P<0.01$ vs. vehicle group, $^{\#}P<0.01$ vs. ovalbumin (OVA) group, $^{\$}P<0.01$ vs. multi-walled nanotubes (MWCNT) group.

Table 3
Protein levels of chemokines in lung tissue supernatants after the final intratracheal challenge.

Treatment	Eotaxin	TARC	MDC	MCP-1	KC
	(pg/total lung tissue supernatants)				
Vehicle	51.8 ± 6.60	37.8 ± 6.39	59.9 ± 6.33	33.4 ± 4.22	51.8 ± 18.0
MWCNT	950.8 ± 99.9**	248.8 ± 11.1**	248.4 ± 16.1**	620.2 ± 46.2**	565.3 ± 50.6**
OVA	1204.6 ± 272.3**	162.8 ± 36.2**	138.6 ± 31.8**	117.5 ± 17.2*	200.3 ± 47.2*
OVA + MWCNT	2061.6 ± 287.6**$	526.7 ± 43.7**#$	410.4 ± 24.3**#$	1617.7 ± 231.8**#$	1151.3 ± 105.2**#$

Results are expressed as the mean ± SE ($n = 8$ in each group). *$P<0.05$ vs. vehicle group, **$P<0.01$ vs. vehicle group, #$P<0.01$ vs. OVA group, $$P<0.05$ vs. MWCNT group, $$$P<0.01$ vs. MWCNT group.

molecules tended to increase after the addition of MWCNT, although the difference did not reach significance.

Effect of MWCNT on antigen-specific syngeneic T-cell-stimulating capacity of BMDC

BMDC function was assessed via their capacity to stimulate antigen-specific syngeneic T-cell proliferation (Fig. 7). The proliferation of T-cells (responder cells) was increased only by the addition of BMDC (stimulator cells). The reaction was significantly increased by exposure to 0.1 μg/ml of MWCNT ($P<0.01$). Treatments with higher concentrations of the nanotubes failed to modulate the activity from levels seen with the untreated cells.

Effects of another type of MWCNT (from SES Research) on allergic airway inflammation and reactivity in vivo

To assess the specificity of MWCNT, we investigated the impacts of other types of MWCNT (50 μg/animal) on the asthma model in the context of BAL cellularity (S-Fig. 1) and specific Ig production (S-Fig. 2). The number of total cells was significantly greater in the OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was greater in the OVA + MWCNT group than in the MWCNT group (N. S.) or OVA group ($P<0.05$). Exposure to OVA or OVA + MWCNT significantly increased the number of eosinophils as compared with vehicle exposure ($P<0.01$). The numbers were significantly greater in the OVA + MWCNT group than in the MWCNT group ($P<0.01$) or the OVA group ($P<0.05$). The number of lymphocytes was greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was greater in the OVA + MWCNT group than in the OVA group ($P<0.01$) or the MWCNT group (N. S.). The number of neutrophils was greater in the MWCNT group, OVA group, or OVA + MWCNT group than in the vehicle group ($P<0.01$). The number was higher in the OVA + MWCNT group than in the OVA group ($P<0.01$) or the MWCNT group (N. S.). The number of macrophages was significantly greater in the OVA + MWCNT group than in the vehicle group, OVA group or MWCNT group ($P<0.01$).

For Ig production, allergen-specific IgG_1 and IgE were significantly greater in the OVA or OVA + MWCNT group than in the vehicle group ($P<0.01$). The titers were further greater in the OVA + MWCNT group than in the OVA group ($P<0.05$) or MWCNT group ($P<0.01$). Accordingly, these results indicate that MWCNT have considerable facilitating properties on OVA-related airway inflammation with specific Ig productions likely regardless of their characteristics (external and internal components).

Table 4
Protein levels of cytokines in bronchoalveolar lavage fluid (BALF) after the final intratracheal challenge.

Treatment	IL-1β	IL-6
	(pg/mg BALF)	
Vehicle	0 ± 0	51.1 ± 7.7
MWCNT	8.5 ± 2.7*	177.8 ± 34.2*
OVA	0 ± 0	37.6 ± 7.8
OVA + MWCNT	32.3 ± 8.1**#$	443.2 ± 79.5**#$

Results are expressed as the mean ± SE ($n = 8$ in each group).
*$P<0.05$ vs. vehicle group, **$P<0.01$ vs. vehicle group,
#$P<0.01$ vs. OVA group, $$P<0.05$ vs. MWCNT group.

Discussion

The present study has shown that MWCNT administered by an intratracheal route deteriorate allergic airway inflammation in mice, which is characterized by the infiltration of inflammatory PMNs in both the bronchoalveolar spaces and lung parenchyma. MWCNT also significantly induce and moderately exacerbate goblet cell metaplasia in the presence or absence of allergen. The enhancing effects are associated with the increased lung levels of proinflammatory cytokines such as IL-1β and IL-6, Th2 cytokines such as IL-4, IL-5, and IL-13, Th2 chemokines such as TARC and MDC, and chemokines related to the migration/maturation of T lymphocytes and neutrophils such as MCP-1 and KC, respectively. Also, MWCNT exhibit adjuvant activity for the allergen-specific production of IgG_1 and IgE. Finally, MWCNT can partially promote the maturation/activation and function of BMDC *in vitro*.

Despite being less studied, we and others have examined the effects of exposure to several types of nanoparticle/material on allergic asthma *in vivo*, showing that carbon black nanoparticles have the potential to advance allergic asthma *in vivo* (de Haar et al., 2005; Inoue et al., 2005). In addition, nano-sized TiO_2 (14–29 nm in size) have reportedly more prominent adjuvant effects on allergen-specific responses with Ig production than fine (250–260 nm in size) ones *in vivo* (de Haar et al., 2006). On the other hand, we have recently found that latex nanoparticles (15–100 nm in size) do not facilitate allergic asthma employing the same protocol as in the present study (Inoue et al., 2009a). These previous observations suggest that each nano-level

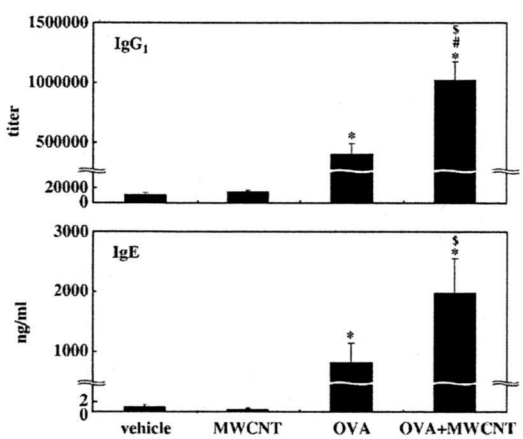

Fig. 5. Allergen-specific IgG_1 and IgE values. Four groups of mice were intratracheally administered vehicle, MWCNT (50 μg/treatment), OVA, or a combination of OVA + MWCNT over a period of 6 wk. Serum samples were retrieved 24 h after the last intratracheal instillation. Antigen-specific IgG_1 and IgE were analyzed using ELISA. Results are expressed as the mean ± SE ($n = 12$–13 in each group). *$P<0.01$ vs. vehicle group, #$P<0.01$ vs. OVA group, $$P<0.01$ vs. MWCNT group.

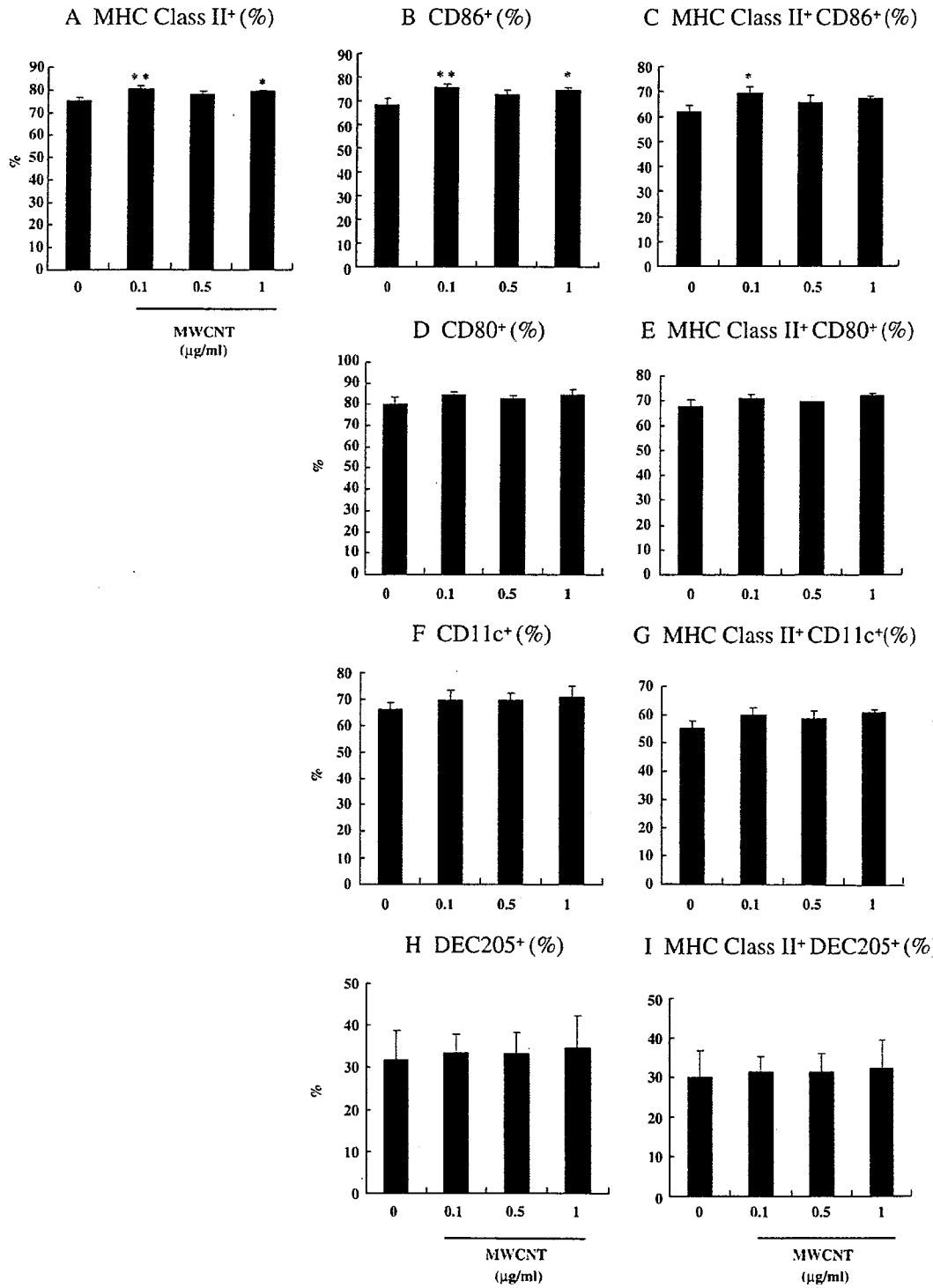

Fig. 6. Effects of MWCNT on the expression of molecules related to antigen presentation in bone marrow-derived dendritic cells (BMDC). Immature BMDC from ICR mice were exposed to MWCNT (0.1–1 μg/ml) as described in Materials and methods. After exposure, the expression of molecules related to antigen presentation was analyzed by flow cytometry. Percentages of each cell type in the BMDC population are shown. (A) MHC Class II$^+$, (B) CD86$^+$, (C) MHC Class II$^+$CD86$^+$, (D) CD80$^+$, (E) MHC Class II$^+$CD80$^+$, (F) CD11c$^+$, (G) MHC Class II$^+$CD11c$^+$, (H) DEC205$^+$, (I) MHC Class II$^+$DEC205$^+$. Data represent the mean ± SE of three animals from one experiment, representative of three experiments ($^*P<0.05$, $^{**}P<0.01$ vs. corresponding control).

particle/material has different effects on this pathology, which may depend on their characteristics. Based on the current data, we propose that MWCNT exposure is a risk factor for the development/aggravation of allergic asthma. Further, enhanced lung expression of allergy-related molecules including IL-4, IL-5, IL-13 (Th2 cytokines), TARC, and MDC (Th2 chemokines) could be important contributors to the

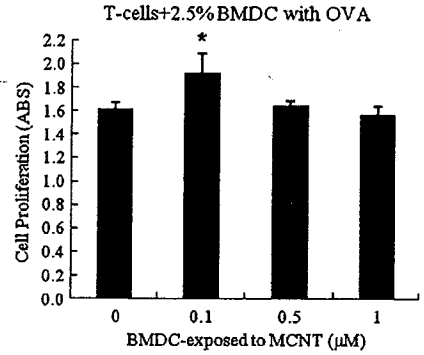

Fig. 7. Effects of MWCNT on antigen-specific syngeneic T-cell-stimulating capacity of BMDC. Immature BMDC from ICR mice were exposed to MWCNT (0.1–1 μg/ml) in the same manner as shown in Fig. 5. Splenic T-cells (2×10^5) from OVA-sensitized ICR mice were co-cultured with BMDC (5×10^3, to be present at 2.5% of the splenic T-cell level) from ICR mice for 4 days. Thereafter, T-cell proliferation was evaluated. Data represent the mean ± SE of three animals from one experiment, representative of two experiments ($^*P<0.05$ vs. corresponding control).

etiology of the aggravation. Regarding the outcome, the unique characteristics of the fibrous shape may be responsible for the allergic asthma-promoting effects, since two kinds of MWCNT showing different characteristics of their components similarly enhance allergic airway inflammation with significance. However, these are not described in reports on the adverse effects of pathogenic fibers such as asbestos on allergic diseases in humans and *in vivo*, although fibrous materials (asbestos and its component silica) have been implicated in the promotion of asthma pathobiology in terms of inducing/enhancing mucin production dependent on the *mclca3* gene (Sabo-Attwood et al., 2005) and local Th2 cytokine expression (Choi et al., 2009) *in vivo*, and IL-4 production with a consequent Th2 response including IgE synthesis in humans (Lange et al., 1995). These implications can be partly linked to our present findings, in which MWCNT alone induced significant goblet cell hyperplasia and amplified IL-5 and IL-13 expressions in the lung. Thus, the (nano-level) material size in addition to its unique (fibrous) shape might be important to cause induction/facilitation in allergic phenotypes. Of course, it is also possible that attached metals and chemical components in these MWCNT potentiated the induction/facilitation in association with their nano-size and unique shape.

The cellular points and underlying molecular mechanistic pathways in which MWCNT potentiate allergic airway inflammation remain obscure. Because the measured cytokine and chemokine levels in lung homogenates (and partially in BALF supernatants) representing products of multiple cell types exist in the distal airways, e.g., macrophages (IL-18, MDC, KC, IL-1β, IL-6), epithelial cells (KC, MCP-1, eotaxin, IL-1β, IL-6), neutrophils (IL-1β, MCP-1), T cells (IL-4, IL-5, IL-13, IL-33, IFN-γ, TARC, MDC, IL-6), fibroblasts (eotaxin, KC, IL-1β, IL-6), and endothelial cells (TARC, KC, IL-1β, IL-6), the data suggest the widespread influence of MWCNT in the presence or absence of allergen. From a few *in vitro* studies, furthermore, CNT has been shown to activate the signaling of mitogen-activated protein kinases, nuclear factor-κB, activator protein-1, and protein serine–threonine kinase, common mechanisms for cytokine expansion *in vitro* (Manna et al., 2005; Pacurari et al., 2008) and *in vivo* (Chou et al., 2008), as is often the case with asbestos (Mossman et al., 1997; Cacciotti et al., 2005). Thus, it appears plausible that the inhaled MWCNT cooperating with allergen synergistically exacerbate allergic inflammation/damage through direct/indirect effects on the local pulmonary microenvironment interacting with these effector cells, possibly through several signaling pathways related to inflammation.

APC including DC, macrophages, and B cells play fundamental roles in the pathogenesis of asthma (Upham, 2003; Kato et al., 2006). APC-mediated phagocyting and subsequent antigen presentation for T-cells is the first step in both primary and secondary immune responses. In particular, DC are recognized to be professional APC, exhibiting a potent antigen-presenting ability (Rossi and Young, 2005). The maturation of DC is an important step for polarized antigen presentation and the consequent Th response (de Jong et al., 2005). We have previously shown that CB nanoparticles activate BMDC employing the same protocol as in the present study *in vitro* (Koike et al., 2008a), and enhance intrapulmonary APC activation (Koike et al., 2008b) commensurate with asthma pathophysiology *in vivo* (Inoue et al., 2005), fully indicating that the effects of nanoparticles on allergic asthma can be mediated, at least in part, through the activation of APC. In addition, we have recently shown that DEP significantly enhance BMDC-mediated antigen-presenting activity at certain concentrations *in vitro* (Inoue et al., 2009b). Furthermore, fibrous particles such as asbestos reportedly activate APC *in vitro* (Hamilton et al., 2004). Taken together, it is likely that inhalable PM or fibrous materials modify APC characterization/function. In the present study, exposure to MWCNT significantly increased the percentage of MHC Class II^+, $CD86^+$, and MHC Class II^+CD86^+ cells, although the response did not clearly exhibit dose-dependency. Furthermore, MWCNT exposure significantly amplified OVA-specific syngeneic T-cell reactivity mediated by BMDC. Therefore, the present *in vitro* results suggest that MWCNT can markedly regulate the phenotypic and functional activation of BMDC at certain doses. In addition, of note, the levels of mediators produced/released by phagocytes (macrophage and dendritic cell: IL-1β) in the BALF supernatants were greater in the OVA + MWCNT group than in the OVA group *in vivo*. Therefore, it is possible that the exacerbating effects of MWCNT on allergic pathophysiology can account for, at least partly, the amplified maturation/activation of APC including DC.

Allergic asthma represents a Th2-dominant response. However, environmental toxicants sometimes deteriorate allergic asthma models not through augmentation of the Th2 response alone, but also through the activation of both Th1 and Th2 responses (Takano et al., 1997; Larsen et al., 2002 and 2004; Yanagisawa et al., 2006; Inoue et al., 2007a). This phenomenon might be explained by their make-up, comprising a complicated mixture of large amounts of organic and inorganic components which stimulate/activate various immune responses. Furthermore, asbestos reportedly can up-regulate both Th1 and Th2 immunity, likely through APC activation *in vitro* (Hamilton et al., 2004). In accordance with these previous findings, in the present study, the combined *in vivo* exposure to OVA + MWCNT profoundly increased the production of both Th1 (IgG_{2a}: data not shown)- and Th2 (IgG_1 and IgE: Fig. 4)-associated Igs as compared with that to OVA alone. Alternate, MWCNT alone might trigger the mixed Th milieu, which subsequently assists in the hyperproduction of allergen-specific Igs, since lung expression levels of IFN-γ as well as IL-5 and IL-13 were elevated by MWCNT exposure in the presence or absence of allergen. On the other hand, a recent report showed that IL-18 and allergen (OVA) activate Th1 cells to produce Th2 cytokines and induce "Th1"-type allergic asthma characterized by the predominance of neutrophilic airway inflammation *in vivo* (Hayashi et al., 2007). Of interest, our recent study has shown that latex nanoparticles amplified lung expressions of IFN-γ (unpublished observation) and IL-18, and subsequent exacerbation neutrophilic airway inflammation related to allergen (OVA) (Inoue et al., 2009a). Similarly in the present study, MWCNT significantly elevated IL-18 expression in the lung in the presence or absence of allergen, aggravated neutrophilic airway inflammation *in vivo*, and tended to increase cytokine production in culture supernatants from MWCNT-exposed BMDC cocultured with T cells *in vitro* (data not shown). Thus, one can lead to the scenario that MWCNT in the presence of allergen independently triggers IL-18 production/release in the airway and subsequent Th1-type airway inflammation, which culminate in the promotion of allergic pathology via a mixed pattern of "Th1/Th2 immune responses", or alternatively, a

"mixed Th response". Nonetheless, to clarify the issue, future systematic investigations should be carried out using *in vitro* and *ex vivo* assays focusing on Th cells.

During the preparation of our manuscript, a study on the effects of MWCNT on another murine asthma model was reported (Ryman-Rasmussen et al., 2009). In their study, MWCNT did not exacerbate allergic airway inflammation with specific Th responses, but augmented airway fibrosis accompanied by inflammation. The results differing from ours, are interesting and may be explained by significant differences from our study regarding many points such as the materials and methods, e.g., characteristics of the MWCNT, mouse strain (C57BL/6), experimental asthma (sensitized and challenged by intraperitoneal injection and inhalation of OVA), MWCNT exposure style (nebulizer, timing), and point of termination (28 days after sensitization). On the other hand, they did not examine the impacts on airway hyperresponsiveness (we examined and found that MWCNT slightly strengthened allergen-related cholinergic airway hyperresponsiveness; data not shown), whereas, we neither compared fibrous changes in the lung structure nor examined local and systemic levels of growth factors including transforming growth factor-β in the current model; thus, one cannot view these two sets of results as opposite outcomes. In other words, taken these positive findings from us and Ryman-Rasmussen's group into consideration, it is proposed that MWCNT can affect/facilitate both "early (induction, effector)" and "late (airway remodeling)" phases of allergic asthma.

In sum, the present study showed that MWCNT can exacerbate allergic airway inflammation with augmented humoral immunity. The enhancing effects are concomitant with the increased lung expression of Th cytokines and chemokines related to inflammatory leukocyte recruitment/infiltration. These results suggest that inhalable MWCNT may become one of the important environmental risk factors of allergic asthma.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.taap.2009.04.003.

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Titanium Dioxide Nanoparticles Aggravate Atopic Dermatitis-Like Skin Lesions in NC/Nga Mice

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Titanium dioxide (TiO_2) nanoparticles are produced abundantly and used ubiquitously in various cosmetic products. However, it remains to be determined whether transdermal exposure to TiO_2 nanoparticles affects atopic dermatitis (AD), which has been increasing in developed countries. We investigated the effects of different sized TiO_2 nanoparticles on AD-like skin lesions induced to mite allergen in NC/Nga mice assumed to show skin barrier dysfunction/defect. Male mice were injected intradermally with TiO_2 nanoparticles of three sizes (15, 50, or 100 nm) and/or mite allergen into their right ears. We evaluated clinical scores, ear thickening, histological findings and the protein expression of T helper (Th) 1 and Th2 cytokines in the ear, and the levels of Ig and histamine in serum. TiO_2 nanoparticles aggravated AD-like skin lesions related to mite allergen in NC/Nga mice. The enhancing effects are paralleled by the overproduction of IL-4 in the skin, the levels of total IgE and histamine in serum regarding the overall trend. In contrast, TiO_2 nanoparticles decreased the local expression of IFN-γ in the presence of allergen. Additionally, TiO_2 nanoparticles alone significantly increased histamine levels in serum and IL-13 expression in the ear. However, different effects related to the size differences of TiO_2 nanoparticles were not observed. In conclusion, exposure to TiO_2 nanoparticles under skin barrier dysfunction/defect can exacerbate AD symptoms through Th2-biased immune responses. Furthermore, TiO_2 nanoparticles can play a significant role in the initiation and/or progression of skin diseases following the barrier dysfunction/defect by histamine release even in the absence of allergen. Exp Biol Med 234:314–322, 2009

Key words: atopic dermatitis; titanium dioxide; eosinophils; mast cells; histamine; IL-4

Introduction

Over the past several decades, the prevalence of atopic dermatitis (AD) has been increasing consecutively in developed countries (1–4). AD is a multifactorial allergic inflammatory skin disorder characterized by chronic pruritic and eczematous lesions, elevated IgE hyperproduction, and infiltrating inflammatory cells, and is generally thought to be a genetic disorder of the immune system, e.g., stratum corneum structural protein and filaggrin (5–9). On the other hand, epidemiological studies have suggested that environmental factors including allergen load, mental stress, and environmental toxicants including chemicals can contribute to the initiation and/or progression of allergic diseases including AD (10, 11). Many animal studies have shown that exposure to various environmental chemicals can aggravate allergic diseases (12–15). Furthermore, environmental chemicals may increase the potency of allergens, and therefore play a crucial role in the enhancement of allergic diseases (16). Consistent with these findings, our recent study has shown that systemic exposure to di-(2-ethylhexyl) phthalate (DEHP), a typical environmental chemical, the most abundant phthalate plasticizer in polyvinylchloride, aggravates AD-like skin lesions related to mite allergen in mice (17). Thus, it is worthwhile to assess whether exposure to environmental chemicals is associated with the increasing prevalence of AD.

Titanium dioxide (TiO_2) is a noncombustible and odorless white powder, which is frequently used as a white pigment in a wide range of paints, paper, ceramics, plastics and food colorants. In particular, nano-sized TiO_2 particles

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(<100 nm) are increasingly used in industrial products including cosmetics and pharmaceuticals. The widespread use of TiO_2 nanoparticles may lead to potential exposure to the engineered nanoparticles in the general environment via respiratory, gastrointestinal, and/or dermal systems.

Generally, nanoparticles, which are characterized by their small size and large surface area, can exhibit more physical and chemical properties than larger ones (i.e., fine or coarse particles). Several studies have demonstrated that exposure to TiO_2 nanoparticles via the respiratory system affects a variety of respiratory diseases such as lung inflammation, pulmonary fibrosis and lung tumors *in vivo* (18–21). We have also reported that TiO_2 nanoparticles exacerbate lung inflammation related to lipopolysaccharide (LPS) (22). Furthermore, our and some other reports have suggested that exposure to carbon nanoparticles contributes to the exacerbation of allergic airway inflammation (23–27). However, there are few reports regarding the effects of exposure to TiO_2 (nano) particles on allergic diseases. De Haar *et al.* reported that intranasal exposure to TiO_2 nanoparticles causes airway inflammation through immune adjuvant activity and Th2 type cytokine production (23, 28). With respect to intradermal exposure, another major exposure route of nanoparticles, TiO_2 nanoparticles do not readily penetrate through the intact epidermal barrier (29–31). However, once AD patients have the barrier dysfunction/defect, TiO_2 nanoparticles may penetrate into the skin, and then affect the cutaneous function. A recent report has shown that a significant increase of *Staphylococcus aureus*, which is thought to play an important role in the exacerbation of AD, can accelerate skin barrier dysfunction induced by anatase-typed TiO_2 nanoparticles (7 nm) and UV irradiation (32). However, most TiO_2 (nano) particles contained in cosmetics are rutile-typed particles which generally occupy 5–10% of sunscreen agent. In addition, TiO_2 nanoparticles in cosmetics can directly contact with the stratum corneum. Recent *in vitro* studies have suggested that TiO_2 particles with different crystal structure show different toxicological responses (33, 34). However, it remains unclear whether transdermal exposure to TiO_2 nanoparticles, especially rutile-typed ones, affects AD with or without skin barrier dysfunction.

The aim of our study was to investigate the effects of rutile-typed TiO_2 nanoparticles with different sizes on AD-like skin lesions related to mite allergen in NC/Nga mice assumed to show skin barrier dysfunction or defect. We also examined the effects following intradermal exposure to TiO_2 nanoparticles alone.

Materials and Methods

Animals. Seven-week-old male NC/NgaTndCrj (NC/Nga) mice were purchased from Charles River Japan (Osaka, Japan). They were fed a commercial diet (Japan Clea Co., Tokyo, Japan) and water ad libitum. Mice were housed in an animal facility that was maintained at 22–26°C with 40–69% humidity and a 12 h/12 h light/dark cycle. All animals used in the research were treated humanely according to guidelines of the National Institute for Environmental Studies for animal experiments, with due consideration to the alleviation of distress and discomfort. All protocols were approved by the Institutional Review Board.

Study Protocol. Eight-week old mice (22–25 g) were divided into 8 experimental groups. TiO_2 nanoparticles were purchased from TAYCA Co., Ltd. (Rutile crystal phase, Osaka, Japan). The size of TiO_2 nanoparticles was 15, 50, or 100 nm (surface area: 110, 20–25, and 10–15 m^2/g, respectively). The vehicle group received 10 μL of saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan). The TiO_2 (15, 50, or 100 nm) groups received 20 μg of TiO_2 nanoparticles of each size in the vehicle. The Dp group received 5 μg of mite allergen extract (*Dermatophagoides pteronyssinus*; Dp, Cosmo Bio LSL, Tokyo, Japan) dissolved in the vehicle. The Dp + TiO_2 15 nm, Dp + TiO_2 50 nm, or Dp + TiO_2 100 nm groups received a combined administration of Dp and 15, 50, or 100 nm TiO_2 nanoparticles, respectively. The TiO_2 nanoparticle suspensions were sonicated for 2 mins with an ultrasonic disruptor (UD-201; Tomy Seiko, Tokyo, Japan) and then mixed together with mite allergen. Mice were injected intradermally with TiO_2 nanoparticles and/or mite allergen on the ventral side of their right ears on days 1, 3, 5, 8, 10, 12, 15, and 17 under anesthesia with 4% halothane (Takeda Chemical Industries, Ltd., Osaka, Japan) and received no treatment on day 0, as previously described (17, 35–37). We measured ear thickness 24 h after each intradermal injection, as previously described (17).

Blood Sampling. Mice were anesthetized with diethyl ether 24 h after the last intradermal injection. The chest and abdominal walls were opened, and blood was sampled by cardiac puncture. Serum was stored at –80°C until being assayed for total IgE, Dp-specific IgG1, and histamine.

Histological Evaluation. The right ears of mice were removed 24 h after the last intradermal injection. Ears were fixed in 10% phosphate-buffered formalin (pH 7.2), embedded in paraffin, cut into 3-μm slices, and stained with hematoxylin and eosin or toluidine blue (pH 4.0). Histological analyses were performed using a microscope (AX80; Olympus, Tokyo, Japan). The length of the cartilages in each specimen was measured by a video micrometer (VM-30; Olympus). The numbers of eosinophils and mast cells in each sample were counted with the micrometer. The infiltration of eosinophils and mast cells were morphometrically evaluated as the number of cells per millimeter of cartilage in a blind fashion. We also evaluated the degranulation of mast cells as: non-degranulated (0%), mildly degranulated (0–50%), and severely degranulated (more than 50%), as previously reported (17).

ELISA. The right ears of mice were removed 24 h after the last intradermal injection. They were homogenized and centrifuged as previously described (38). ELISA for IL-

4 (Amersham, Buckinghamshire, UK). IL-5 (Endogen, Cambridge, MA), IL-13 (R&D Systems, Minneapolis, MN), IFN-γ (Endogen), and IL-12/IL-23 p40 (R&D Systems) in the ear tissue supernatants were conducted according to the manufacturers' instructions. The detection limits of these assays were <5, 5, 1.5, 10, and 4 pg/ml, respectively.

Dp-specific IgG1 antibodies in serum were measured by ELISA with solid-phase antigen, as previously described (39). Total IgE antibodies and histamine in serum were measured by OptEIA™ Set Mouse IgE (BD Biosciences, San Diego, CA, USA) and HISTAMINE ENZYME IMUUNOASSAY KIT (SPI-BIO, Montigny le Bretonneux, France) according to the manufacturers' instructions, respectively.

Statistical Analysis. Data are reported as the mean ± SE. The significance of variation among different groups was determined by one-way ANOVA or Kruskal-Wallis analysis. Differences between experimental group and control group were determined by Dunnett's or steel multiple comparison test. P value of less than 0.05 was considered to be significant. We used statistical software (Stat View version 5.0; Abacus Concepts, Inc., Berkeley, CA).

Results

Effects of TiO_2 Nanoparticles on AD-Like Skin Lesions. To evaluate the effects of TiO_2 nanoparticles on the skin in the presence or absence of mite allergen, we examined ear thickening and clinical scores 24 h after each intradermal injection. The intradermal administration of vehicle or TiO_2 nanoparticles did not affect the ear thickening (Fig. 1) nor clinical scores (data not shown). Treatment with mite allergen significantly enhanced ear thickening as compared with vehicle treatment from day 3 after the first intradermal injection ($P < 0.01$). Furthermore, the combined administration of mite allergen and TiO_2 nanoparticles significantly enhanced ear thickening as compared with mite allergen administration alone ($P < 0.05$). This result was paralleled by the clinical scores including dryness, wound severity, and edema. However, the size differences of TiO_2 nanoparticles did not affect ear thickening and clinical scores in the presence of mite allergen.

Effects of TiO_2 Nanoparticles on Histological Changes in the Skin. To evaluate histological changes, we performed hematoxylin and eosin (Fig. 2A–D) and toluidine blue staining (Fig. 2E–H) 24 h after the last intradermal injection. Exposure to vehicle (Fig. 2B, F) or TiO_2 nanoparticles did not lead to significant pathologic alterations. Intradermal injection of mite allergen caused eosinophilic infiltration into the skin as compared with vehicle injection (Fig. 2A, C: $P < 0.05$). Furthermore, the combined administration of mite allergen and TiO_2 nanoparticles increased the number of eosinophils as compared with mite allergen administrated alone (Fig. 2A, D: $P < 0.05$ for Dp + TiO_2 treated groups vs. Dp group). A similar trend was observed regarding the severity of mast cell degranulation (Fig. 2E–H). However, the size differences in TiO_2 nanoparticles did not affect histological changes in mite allergen presence in our model (Fig. 2A, E).

Effects of TiO_2 Nanoparticles on the Protein Expression of Proinflammatory Molecules in the Skin. We investigated the effects of TiO_2 nanoparticles on the protein expression of Th1 and Th2 cytokines in the ear 24 h after the last intradermal injection (Table 1). The protein levels of IL-4, IL-5, and IL-13 in the Dp groups were significantly greater than those in the vehicle group (IL-4, $P < 0.05$; IL-5, $P < 0.01$; IL-13, $P < 0.01$, respectively). Additionally, the administration of 15 and 50 nm TiO_2 nanoparticles combined with mite allergen led to a further significant increase in IL-4 as compared with mite allergen administration alone ($P < 0.05$). Furthermore, intradermal exposure to TiO_2 nanoparticles significantly elevated the expression of IL-13 in the absence of mite allergen as compared with vehicle exposure ($P < 0.05$). On the other hand, mite allergen treatment in the presence and absence of TiO_2 nanoparticles significantly decreased the expression of IFN-γ as compared with vehicle treatment ($P < 0.05$). In addition, the administration of 15 or 50 nm TiO_2 nanoparticles combined with mite allergen further decreased IFN-γ production compared with mite allergen administration alone ($P < 0.05$). IL-12/IL-23 p40 was not detectable among all experimental groups.

Effects of TiO_2 Nanoparticles on Ig Levels in Serum. To evaluate the adjuvant activity of exposure to TiO_2 nanoparticles for Ig production, we measured total IgE and allergen-specific IgG1 in serum 24 h after the last intradermal injection. Mite allergen administration significantly increased the levels of total IgE and allergen-specific IgG1 as compared with vehicle administration (Fig. 3; $P < 0.01$). There was a trend toward higher levels of Ig production in the Dp + TiO_2 groups than in the Dp group. In particular, combined treatment with mite allergen and 50 nm TiO_2 nanoparticles resulted in a further significant increase in total IgE as compared to treatment with mite allergen alone ($P < 0.05$).

Effects of TiO_2 Nanoparticles on Histamine Levels in Serum. To evaluate the effects of exposure to TiO_2 nanoparticles on histamine release in the presence or absence of allergen, we measured histamine levels in serum 24 h after the last intradermal injection. Mite allergen or TiO_2 treatment elevated the levels of histamine in serum as compared with vehicle treatment (Fig. 4, $P < 0.01$). Additionally, combined administration of mite allergen and TiO_2 nanoparticles showed a further significant increase in histamine release as compared with mite allergen administration alone ($P < 0.01$).

Discussion

The present study showed that intradermal injection of TiO_2 nanoparticles could aggravate AD-like skin lesions

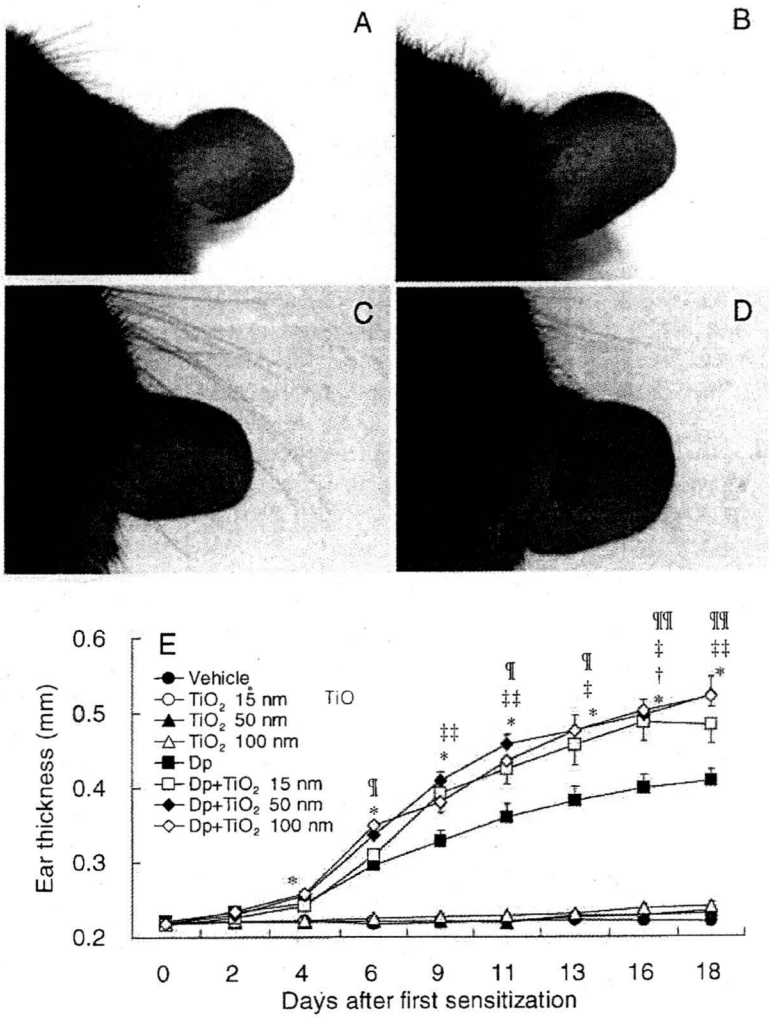

Figure 1. Effects of TiO_2 nanoparticles on ear thickening of AD-like skin lesions induced by mite allergen (Dp). We showed macroscopic features (A–D) and measured ear thickening (E) 24 h after each intradermal Dp injection. (A): vehicle group, (B): TiO_2 50nm group, (C): Dp group, (D): Dp+TiO_2 50 nm group. Data are the means ± SE of 11 animals per group. * $P < 0.01$ Dp-treated groups vs. vehicle group. † $P < 0.05$ Dp+TiO_2 15 nm group vs. Dp group. †† $P < 0.01$ Dp+TiO_2 15 nm group vs. Dp group. ‡ $P < 0.05$ Dp+TiO_2 50 nm group vs. Dp group. ‡‡ $P < 0.01$ Dp+TiO_2 50 nm group vs. Dp group. ¶ $P < 0.05$ Dp+TiO_2 100 nm group vs. Dp group. ¶¶ $P < 0.01$ Dp+TiO_2 100 nm group vs. Dp group.

related to mite allergen in NC/Nga mice assumed to show skin barrier dysfunction/defect. These results were paralleled by the overproduction of IL-4 in the skin, the levels of total IgE and histamine in serum regarding the overall trend. In contrast, the administration of TiO_2 nanoparticles combined with mite allergen decreased the local expression of IFN-γ.

TiO_2 nanoparticles are produced abundantly and used ubiquitously because of their marked stability, as well as activities as anticorrosion and photocatalysis. However, the potential toxicity remains unclear. Several studies have demonstrated that exposure to TiO_2 nanoparticles affects a variety of respiratory diseases including pulmonary inflammation, pulmonary fibrosis, and lung tumors *in vivo* (18–21). Recently, we have also reported that intratracheal exposure to TiO_2 nanoparticles exacerbates acute lung inflammation related to LPS (22). On the other hand, the effects of TiO_2 nanoparticles on the skin, another major route of exposure other than the respiratory system, remains poorly determined, although they can serve directly as a physical photoprotective reagent in sunscreen and are widely used in various cosmetic products. In particular, sunscreens generally include about 5–10% of TiO_2 (nano) particles and we routinely use 100 μL of sunscreen agent (equivalent to 5–10 mg TiO_2 (nano) particles) on the face each use.

Recent studies have demonstrated that skin barrier dysfunction/defect can be responsible for the pathogenesis of AD in both a murine model and in human patients (40, 41). In our present study, intradermal injection of TiO_2

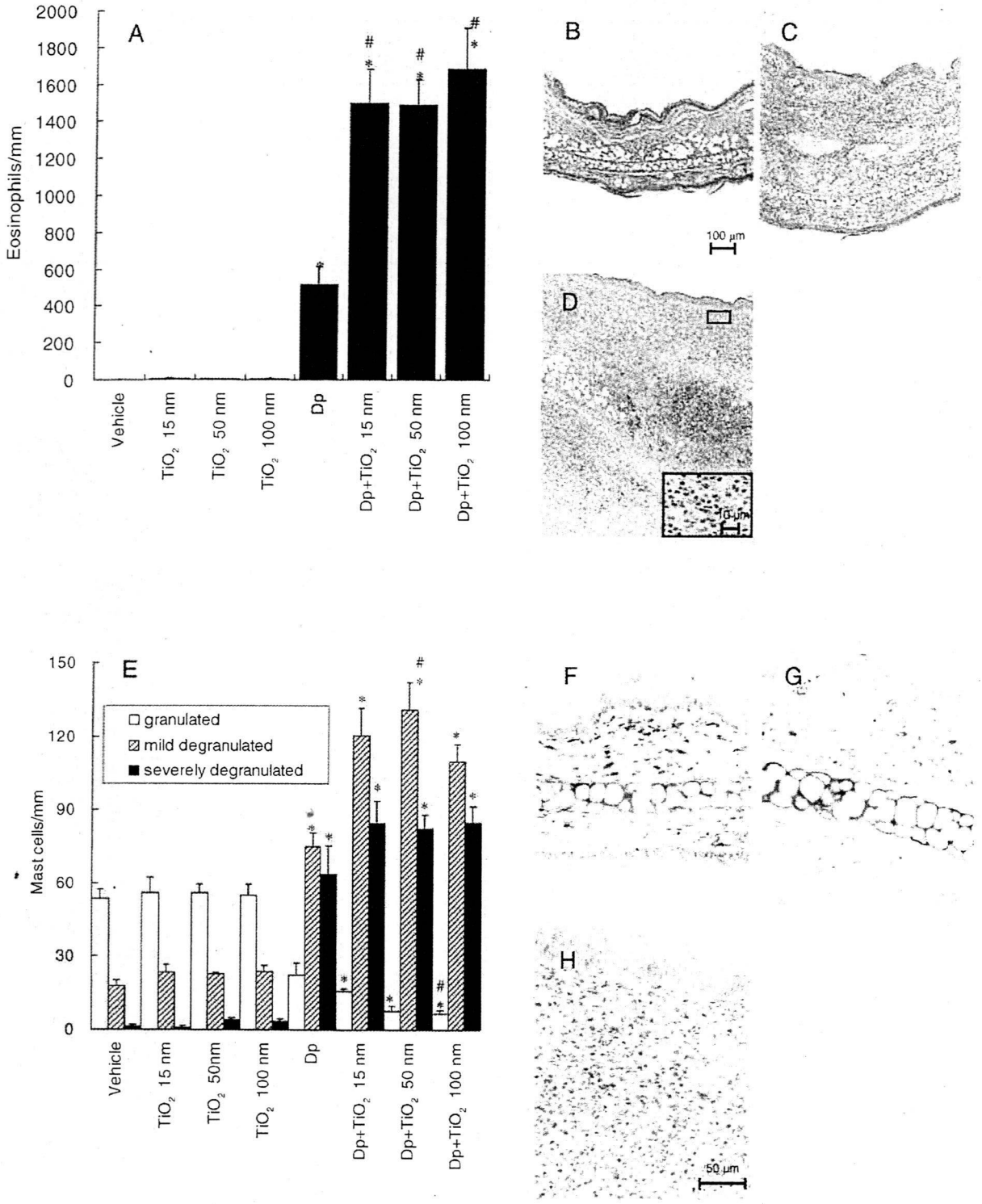

Figure 2. Effects of TiO_2 nanoparticles on histological changes in the ear. Right ears of mice were removed 24 h after the last intradermal injection. The infiltration of eosinophils (A) and the degranulation of mast cells (E) were morphometrically evaluated as the number of cells per millimeter of cartilage. Histological findings of the vehicle group (B, F), Dp group (C, G), or Dp+TiO_2 50 nm group (D, H) are shown with hematoxylin and eosin staining (B–D) or toluidine blue staining (F–H). Data are the mean ± SE of 4 animals per group. Sections were observed at magnifications of ×100, ×200, and ×400. * $P < 0.05$ vs. vehicle group. # $P < 0.05$ vs. Dp group.

Table 1. Effects of TiO_2 Nanoparticles on the Protein Expression of Th1 and Th2 Cytokines in the Ear[a]

Group	Th1 cytokines		Th2 cytokines		
	IFN-γ[b]	IL-12/IL-23 p40[b]	IL-4[b]	IL-5[b]	IL-13[b]
Vehicle	487 ± 18.2		0	0	0
TiO_2 15 nm	432 ± 31.2		0	0	3.09 ± 0.72**
TiO_2 50 nm	402 ± 51.7		0	0	2.50 ± 0.95*
TiO_2 100 nm	365 ± 20.4	ND	0	0	2.84 ± 0.52**
Dp	185 ± 19.3*		8.48 ± 1.93*	4.55 ± 1.28**	8.73 ± 0.64**
Dp+TiO_2 15 nm	80.9 ± 9.67*,#		32.9 ± 7.58**,#	2.15 ± 0.58**	8.27 ± 0.60**
Dp+TiO_2 50 nm	83.8 ± 16.6*,#		39.5 ± 8.21**,#	3.30 ± 0.95**	9.07 ± 1.24**
Dp+TiO_2 100 nm	105 ± 17.7*		30.3 ± 9.14**	2.36 ± 0.45**	7.54 ± 0.77**

[a] Right ears of mice were removed 24 h after the last intradermal injection. Protein levels of cytokines in the ear tissue were analyzed using ELISA. TiO_2, titanium dioxide; Th, T helper; IFN, interferon; IL, interleukin; Dp, *Dermatophagoides pteronyssinus*; ND, not detected.
[b] Results expressed in pg/mg total protein.
* $P < 0.05$ vs. vehicle group.
** $P < 0.01$ vs. vehicle group.
# $P < 0.05$ vs. Dp group. Data are the means ± SEM of 6–7 animals per group.

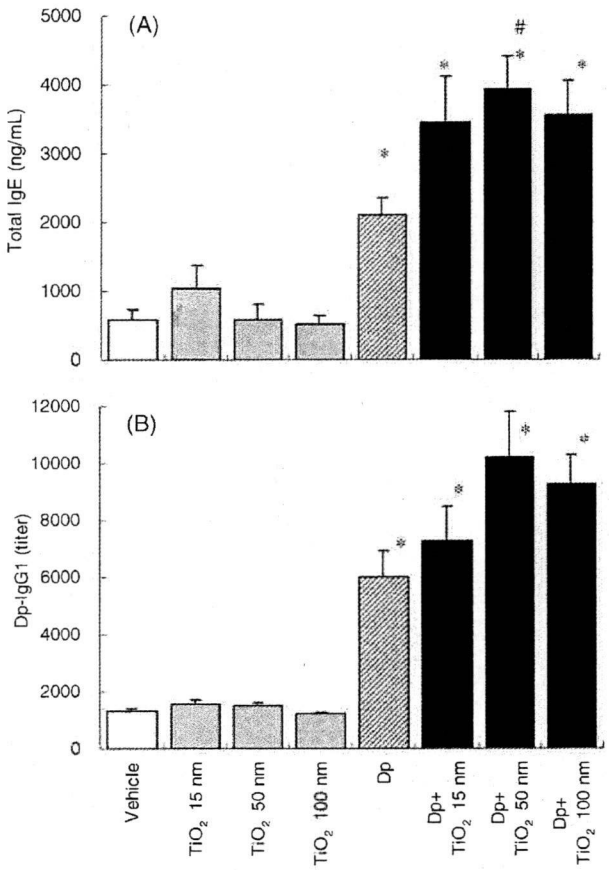

Figure 3. Effects of TiO_2 nanoparticles on Ig production in serum. To evaluate the adjuvant activity of TiO_2 nanoparticles, we measured total IgE and Dp-specific IgG1 in serum 24 h after the last intradermal injection using ELISA. Data are the mean ± SE of 11 animals per group. * $P < 0.01$ vs. vehicle group. # $P < 0.05$ vs. Dp group.

nanoparticles into the skin aggravated AD-like skin lesions induced by mite allergen in a mouse model. Almost all previous studies have demonstrated that TiO_2 nanoparticles *in vivo* do not readily penetrate through the intact epidermal barrier and remain in the stratum corneum (29–31). In a minority of subjects, the presence of TiO_2 nanoparticles was observed in the hair follicles (42). On the other hand, once TiO_2 nanoparticles penetrate into the skin, they can affect the cutaneous function. Kambara *et al.* reported that anatase-typed TiO_2 nanoparticles can accelerate skin barrier dysfunction/defect induced by UV irradiation in mice (32). Additionally, fluoresceinated quantum dots following intradermal injection (15–20 nm) have been shown to migrate to local lymph nodes in mice and pigs (43). It is likely that TiO_2 nanoparticles after transdermal exposure can transfer to local lymph nodes and thereby affect biological and/or immune responses including AD. When administrated directly to cell culture *in vitro*, TiO_2 nanoparticles can exert a variety of adverse effects. Ambient nanoparticles (manufactured TiO_2, carbon black, and polystyrene) were capable of inducing cellular ROS production (44). TiO_2 treatment catalyzes DNA damage in human cells (45) and induces apoptosis in Syrian hamster embryo fibroblasts (46). In addition, Wottrich *et al.* reported that TiO_2 nanoparticles increase inflammatory cytokine release such as IL-6 and IL-8 from epithelial cells (47). Actually, the present *in vivo* study demonstrated that intradermal TiO_2 nanoparticles in the presence of allergen could contribute to the aggravation of AD, a multifactorial allergic skin disorder.

Nano-sized particles are diffusing into the environment with the increasing development of nanotechnology. Their small size and large surface area can contribute to intrinsic toxicity. Concerning TiO_2 toxicity, intratracheal instillation and inhalation exposure to TiO_2 (nano) particles enhance the infiltration of inflammatory cells into the lung and histological changes in animal models (19, 20, 48, 49).

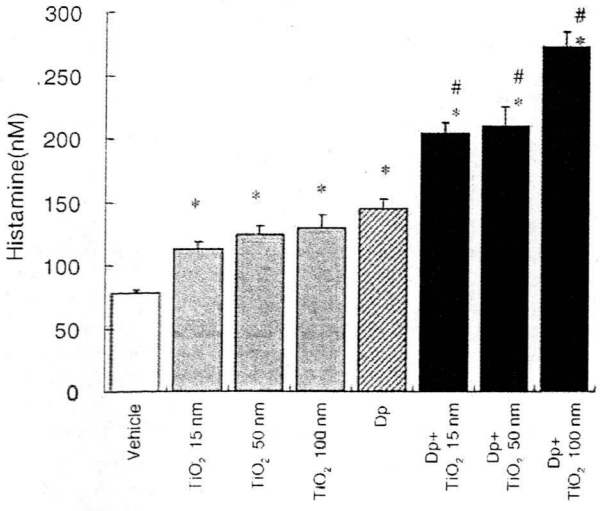

Figure 4. Effects of TiO_2 nanoparticles on histamine release in serum 24 h after the last intradermal injection. Histamine levels in serum were measured using ELISA. Data are the mean ± SE of 11 animals per group. * $P < 0.01$ vs. vehicle group. # $P < 0.01$ vs. Dp group.

Oberdorster and his colleagues have shown that a pulmonary inflammatory response occurs only for 20 nm TiO_2 particles, but not for 250 nm (50). Ultrafine TiO_2 particles (29 nm) were more potent than fine particles (250 nm) regarding airway inflammation related to allergen (28). Furthermore, our recent study showed that intratracheal exposure to TiO_2 nanoparticles exacerbates lung inflammation related to LPS, which is more prominent with smaller nanoparticles than with larger ones. On the other hand, several reports have suggested that the toxicity of particles is not correlated with the size (51, 52). In our present murine model, size-related effects were not observed, which could not be considered to be due to differences in the absorption/penetration of nanoparticles because of the injection directly into the skin. Several previous studies have used the skin-stripping methods as a model of skin barrier dysfunction/defect in humans (53, 54) and experimental animals (55, 56). However, it is difficult to generate quantitatively skin barrier dysfunction/defect with identical severity. Thus, we should try to investigate the size-related effects of TiO_2 nanoparticles using other exposure methods which assume exposure in the "real world" in the future. Nonetheless, our present study suggests that particle sizes may be independent of the severity of AD-like skin lesions in the presence of intradermal TiO_2 nanoparticles of an equal quantity.

Skin lesions in AD are characterized by the recruitment of lymphocytes, monocytes/macrophages, and eosinophils, and the infiltration/degranulation of mast cells. The lymphocytes infiltrating into skin lesions of AD are Th2-type T cells, which produce IL-4, IL-5, and IL-13 (57, 58). On the other hand, defective IFN-γ (a Th1 cytokine) production is considered to be associated with allergen-specific Th2 immune responses in AD patients (59–61). Th2 cytokines play an important role in the pathogenesis of AD-like skin lesions in murine models as well as in that of human AD (40, 41). IL-4 is a crucial factor in IgE synthesis, and IL-5 is also a key factor in the activation and/or proliferation of eosinophils. In *ex vivo* analyses, the protein and mRNA levels of IL-4, IL-5, and IL-13 in spleen or lymph node cells are paralleled by AD-like skin legions in NC/Nga mice (62, 63). The intranasal administration of TiO_2 combined with allergen increase Th2 cytokines (IL-4, IL-5, and IL-13), and allergen-specific IgE and IgG1 levels in serum (28). In the present study, the local protein expression of IL-4, IL-5, and IL-13 in the ear elevated following exposure to mite allergen. In addition, the administration of TiO_2 nanoparticles combined with mite allergen significantly increased IL-4 production as compared with mite allergen administration alone, which was concomitant with the histological changes including the accumulation of eosinophils in the skin and the infiltration/degranulation of mast cells accompanied by histamine release in serum. On the other hand, treatment with mite allergen reduced the expression of IFN-γ, which was more prominent in the Dp + TiO_2 group than in the Dp group. These results and the previous reports suggest that intradermal exposure to TiO_2 nanoparticles can accelerate AD-like skin lesions, possibly via Th2-skewed immune responses.

The degranulation of mast cells on allergen-specific or nonspecific stimulation induces the release of several chemical mediators including histamine. In patients with AD, increases in histamine levels have been observed (64, 65). Our previous study demonstrated that the degranulation of mast cells in the skin is concomitant with the severity of AD-like skin lesions in a murine model (17). Chymase, which is a specific enzyme of connective tissue mast cells, may contribute to the development of atopic inflammation (66). Mast cell degranulation and consequent histamine release can play an important role in the aggravation of AD-like skin lesions. In the present study, intradermal exposure to nanoparticles enhanced histamine release in serum in the presence of allergen. More interestingly, TiO_2 nanoparticles significantly increased histamine release in serum and IL-13 production in the ear in spite of the absence of mite allergen (Table 1, Fig. 4). In addition, the degranulation of mast cells slightly increased following the administration of TiO_2 nanoparticles alone (Fig. 2). Th2 cells as well as mast cells generate IL-13. Thus, TiO_2 nanoparticles might be an important contributor to cutaneous abnormalities with or without allergen under skin barrier dysfunction/defect through chemical mediators derived from mast cells.

In conclusion, intradermal exposure to TiO_2 nanoparticles can aggravate AD-like skin lesions related to mite allergen in our murine model. The effects are paralleled by the enhancement of IL-4 expression in the ear, the levels of total IgE and histamine in serum, and the reduction of IFN-γ expression. Taken together, TiO2 nanoparticles under skin barrier dysfunction/defect may aggravate AD symptoms via Th2-biased immune responses.

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