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# Involvement of macrophage inflammatory protein 1α (MIP1α) in promotion of rat lung and mammary carcinogenic activity of nanoscale titanium dioxide particles administered by intra-pulmonary spraying

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**Titanium dioxide ($TiO_2$) is evaluated by World Health Organization/International Agency for Research on Cancer as a Group 2B carcinogen. The present study was conducted to detect carcinogenic activity of nanoscale $TiO_2$ administered by a novel intrapulmonary spraying (IPS)-initiation–promotion protocol in the rat lung. Female human c-Ha-*ras* proto-oncogene transgenic rat (H*ras*128) transgenic rats were treated first with *N*-nitrosobis(2-hydroxypropyl)amine (DHPN) in the drinking water and then with $TiO_2$ (rutile type, mean diameter 20 nm, without coating) by IPS. $TiO_2$ treatment significantly increased the multiplicity of DHPN-induced alveolar cell hyperplasias and adenomas in the lung, and the multiplicity of mammary adenocarcinomas, confirming the effectiveness of the IPS-initiation–promotion protocol. $TiO_2$ aggregates were localized exclusively in alveolar macrophages and had a mean diameter of 107.4 nm. To investigate the underlying mechanism of its carcinogenic effects, $TiO_2$ was administered to wild-type rats by IPS five times over 9 days. $TiO_2$ treatment significantly increased 8-hydroxydeoxy guanosine level, superoxide dismutase activity and macrophage inflammatory protein 1α (MIP1α) expression in the lung. MIP1α, detected in the cytoplasm of $TiO_2$-laden alveolar macrophages *in vivo* and in the media of rat primary alveolar macrophages treated with $TiO_2$ *in vitro*, enhanced proliferation of human lung cancer cells. Furthermore, MIP1α, also detected in the sera and mammary adenocarcinomas of $TiO_2$-treated H*ras*128 rats, enhanced proliferation of rat mammary carcinoma cells. These data indicate that secreted MIP1α from $TiO_2$-laden alveolar macrophages can cause cell proliferation in the alveoli and mammary gland and suggest that $TiO_2$ tumor promotion is mediated by MIP1α acting locally in the alveoli and distantly in the mammary gland after transport via the circulation.**

## Introduction

Inhalation of particles and fiber is well known to be strongly associated with increased lung cancer risk in the workplace (1,2). Although the size of fiber particles was reported to be closely related to risk (3), the precise role of particles and fibers in lung cancer induction has not yet been elucidated.

Titanium dioxide ($TiO_2$) particles of various sizes are manufactured worldwide in large quantities and are used in a wide range of applications. $TiO_2$ particles have long been considered to pose little risk to respiratory health because they are chemically and thermally stable. However, $TiO_2$ is classified as a Group 2B carcinogen, a possible carcinogen to humans, by World Health Organization/International Agency for Research on Cancer based on the findings of lung tumor induction in female rats (3,4). This overall evaluation includes nanoscale (<100 nm in diameter) and larger sized classes of $TiO_2$. At present, the mechanism underlying the development of rat lung tumors by inhalation of $TiO_2$ particles is unclear.

Inhalation of $TiO_2$ particles can occur both at the workplace, e.g. in manufacturing and packing sites, and also outside the workplace during their use (5–7). Exposure to airborne nanoparticles has been reported to be associated with a granulomatous inflammatory response in the lung (8). Inhalation studies of nanoparticles for cancer risk assessment is urgently needed, however, due to the high cost of long-term studies, available data is severely limited (9,10). The aim of this study is to understand the mechanism underlying rat lung carcinogenesis induced by inhalation of $TiO_2$ particles. We choose intrapulmonary spraying (IPS) because it does not require costly facilities, allows accurate dose control and approximates long-term inhalation studies (3,11).

We initially examined whether $TiO_2$ particles have carcinogenic activity in the rat lung using a novel IPS-initiation–promotion protocol (12,13). For these experiments, Sprague–Dawley (SD)-derived female human c-Ha-*ras* proto-oncogene transgenic rat (H*ras*128) transgenic rats, which are known to have the same carcinogen susceptibility phenotype in the lung as wild-type rats but are highly susceptible to mammary tumor induction (14–16), were treated with *N*-nitrosobis(2-hydroxypropyl)amine (DHPN) to initiate carcinogenesis and then treated with $TiO_2$ by IPS. We observed a promotion effect of $TiO_2$ particles in lung and mammary gland carcinogenesis.

To identify factors involved in this promotion effect, wild-type SD strain rats were treated with $TiO_2$ by IPS for 9 days. We found macrophage inflammatory protein 1α (MIP1α) was produced by $TiO_2$-laden alveolar macrophages in the lungs of rats treated with $TiO_2$. MIP1α is a member of the CC chemokine family and is primarily associated with cell adhesion and migration of multiple myeloma cells (17). It is reported to be produced by macrophages in response to a variety of inflammatory stimuli including $TiO_2$ (18). In the present study, MIP1α, detected in the medium of rat primary alveolar macrophages treated with $TiO_2$, enhanced proliferation of human lung cancer cells *in vitro*. MIP1α was also detected in the sera and mammary adenocarcinomas of $TiO_2$-treated H*ras*128 rats and enhanced proliferation of rat mammary carcinoma cells.

## Materials and methods

### *Animals*

Female transgenic rats carrying the H*ras*128 and female wild-type SD rats were obtained from CLEA Japan Co., Ltd (Tokyo, Japan) (15). The animals were housed in the animal center of Nagoya City University Medical School, maintained on a 12 h light–dark cycle and received Oriental MF basal diet (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. The research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of

**Abbreviations:** CCR1, C-C chemokine receptor type 1; DHPN, *N*-nitrosobis (2-hydroxypropyl) amine; ERK, extracellular signal-regulated kinase; GRO, growth-regulated oncogene; H*ras*128 rat, human c-Ha-*ras* proto-oncogene transgenic rat; IL, interleukin; IPS, intrapulmonary spraying; MEK1, MAPK/ERK kinase1; MIP1α, macrophage inflammatory protein 1α; 8-OHdG, 8-hydroxydeoxy guanosine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SD, Sprague–Dawley; SOD, superoxide dismutase; TEM, transmission electron microscopy; $TiO_2$, titanium dioxide.

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Nagoya City University Medical School and the experimental protocol was approved by the Institutional Animal Care and Use Committee (H17-28).

*Preparation of $TiO_2$ and IPS*

$TiO_2$ particles (rutile type, without coating; with a mean primary size of 20 nm) were provided by Japan Cosmetic Association, Tokyo, Japan. $TiO_2$ particles were suspended in saline at 250 μg/ml or 500 μg/ml. The suspension was autoclaved and then sonicated for 20 min just before use. The $TiO_2$ suspension was intratracheally administered to animals under isoflurane anesthesia using a Microsprayer (Series IA-1B Intratracheal Aerosolizer, Penn-Century, Philadelphia, PA) connected to a 1 ml syringe; the nozzle of the sprayer was inserted into the trachea through the larynx and a total of 0.5 ml suspension was sprayed into the lungs synchronizing with spontaneous respiratory inhalation (IPS).

*IPS-initiation–promotion protocol*

Thirty-three female H*ras*128 rats aged 6 weeks were given 0.2% DHPN (Wako Chemicals Co., Ltd Osaka, Japan) in the drinking water for 2 weeks and 9 rats were given drinking water without DHPN. Two weeks later, the rats were divided into four groups, DHPN alone (Group 1), DHPN followed by 250 μg/ml $TiO_2$ (Group 2), DHPN followed by 500 μg/ml $TiO_2$ (Group 3) and 500 μg/ml $TiO_2$ without DHPN (Group 4). The $TiO_2$ particle preparations were administered by IPS once every 2 weeks from the end of week 4 to week 16 (a total of seven times). The total amount of $TiO_2$ administered to Groups 1, 2, 3 and 4 were 0, 0.875, 1.75 and 1.75 mg per rat, respectively. Three days after the last treatment, animals were killed and the organs (brain, lung, liver, spleen, kidney, mammary gland, ovaries, uterus and neck lymph nodes) were excised and divided into two pieces; one piece was immediately frozen at −80°C and used for quantitative measurement of elemental titanium, and the other piece was fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS) buffer adjusted to pH 7.3 and processed for light microscopic examination and transmission electron microscopy (TEM); the left lungs and inguinal mammary glands were used for elemental titanium analysis and the right lungs and inguinal mammary glands were used for microscopic examination.

*IPS 9 day protocol*

Twenty female SD rats (wild-type counterpart of H*ras*128) aged 10 weeks were treated by IPS with 0.5 ml suspension of 500 μg/ml $TiO_2$ particles in saline five times over a 9 day period (Figure 2A). The total amount of $TiO_2$ administered was 1.25 mg per rat. Six hours after the last dose, animals were killed and the lungs and inguinal mammary glands were excised. Fatty tissue surrounding the mammary gland was removed as much as possible. The left lungs and inguinal mammary glands were used for biochemical analysis, and the right lungs were fixed in 4% paraformaldehyde solution in PBS adjusted at pH 7.3 and processed for histopathological examination and immunohistochemistry.

*Light microscopic and TEM observation of $TiO_2$ particles in the lung*

Paraffin blocks were deparaffinized and embedded in epon resin and processed for $TiO_2$ particle observation and titanium element analysis, using a JEM-1010 transmission electron microscope (JEOL Co. Ltd, Tokyo, Japan) equipped with an X-ray microanalyzer (EDAX, Tokyo, Japan). Size analysis of $TiO_2$ particles was performed using TEM photos by an image analyzer system, (IPAP, Sumika Technos Corporation, Osaka, Japan). A total of 452 particles from alveolar macrophages from rats in Group 3 (DHPN followed by 500 μg/ml $TiO_2$) of the IPS-initiation–promotion study and a total of 2571 particles from alveolar macrophages from rats in the IPS 9 day study were measured.

*Biochemical element analysis of titanium*

For the detection of elemental titanium, frozen tissue samples of 50–100 mg were digested with 5 ml concentrated $HNO_3$ for 22 min in a microwave oven. Titanium in the digested solutions was determined by inductively coupled plasma–mass spectrometry (HP-4500, Hewlett-Packard Co., Houston, TX) under the following conditions: RF power, 1450 W; RF refraction current, 5 W; Plasma gas current, 15 l/min; Carrier gas current, 0.91 l/min; Peri pump, 0.2 r.p.s.; Monitoring mass-m/z 48 (Ti); Integrating interval, 0.1 s; Sampling period 0.31 s.

*Analysis of superoxide dismutase activity, 8-hydroxydeoxy guanosine and cytokine levels*

For the analysis of superoxide dismutase (SOD) activity, 8-hydroxydeoxy guanosine (8-OHdG) and cytokine levels, animals exposed to $TiO_2$ particles for 9 days were used. For 8-OHdG levels, genomic DNA was isolated from the left lung and inguinal mammary gland with a DNA Extractor WB Kit (Wako Chemicals Co. Ltd). 8-OHdG levels were determined with an 8-OHdG ELISA Check Kit (Japan Institute for the Control of Aging, Shizuoka, Japan) and by a custom service (OHG Institute Co., Ltd, Fukuoka, Japan). For the analysis of SOD activity and inflammation-related cytokines, tissue from the left lung and inguinal mammary glands was excised and rinsed with cold PBS three times and homogenized in 1 ml of T-PER, Tissue Protein Extraction Reagent (Pierce, Rockford, IL), containing 1% (vol/vol) proteinase inhibitor cocktail (Sigma–Aldrich, St Louis, MO). The homogenates were clarified by centrifugation at 10 000*g* for 5 min at 4°C. Protein content was measured using a BCA™ Protein Assay Kit (Pierce). SOD activity was determined using an SOD Assay Kit (Cayman Chemical Co., Ann Arbor, MI). The levels of interleukin (IL)-1α, IL-1β, IL-6, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, tumor necrosis factor α, interferon γ, IL-18, monocyte chemotactic protein 1 and MIP1α, growth-regulated oncogene (GRO) and vascular endothelial growth factor were measured by Multiplex Suspension array (GeneticLab Co., Ltd, Sapporo, Japan).

*Immunohistochemistry*

CD68 and MIP1α were detected using anti-rat CD68 (BMA Biomedicals, Augst, Switzerland) and anti-rat MIP1α polyclonal antibodies (BioVision, Lyon, France). Both antibodies were diluted 1:100 in blocking solution and applied to slides, and the slides were incubated at 4°C overnight. The slides were then incubated for 1 h with biotinylated species-specific secondary antibodies diluted 1:500 (Vector Laboratories, Burlingame, CA) and visualized using avidin-conjugated alkaline phosphatase complex (ABC kit, Vector Laboratories) and Alkaline Phosphatase Substrate Kit (Vector Laboratories).

*Isolation of primary alveolar macrophages and preparation of conditioned media*

Wild-type female SD rats were given 0.5 ml 6% thioglycollate medium (Thioglycollate Medium II, Eiken Chemical Co., Ltd, Tokyo, Japan) by IPS on days 1, 3 and 5, and 6 h after the last treatment, the lungs were excised and minced with sterilized scissors in RPMI 1640 containing 10% fetal bovine serum (Wako Chemicals Co., Ltd) and antibiotics. The homogenate was washed twice and plated onto 6 cm dishes and incubated for 2 h at 37°C, 5% $CO_2$. The dishes were then washed with PBS three times to remove unattached cells and cell debris. Samples of the remaining adherent cells were cultured in chamber slides and immunostained for CD68 to confirm their identity as macrophages; ~98% of the cells were positive for CD68.

Primary alveolar macrophages were treated with vehicle or $TiO_2$ particles in saline suspension at a final concentration of 100 μg/ml and then incubated for 24 h in a 37°C, 5% $CO_2$ incubator. The conditioned medium was collected and diluted 5-fold with RPMI 1640; the conditioned medium had a final concentration of 2% fetal bovine serum.

*Western blotting*

For the detection of MIP1α, aliquots of 20 μg protein from the extracts of lung or mammary tissue were separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted. For the detection of C-C chemokine receptor type 1 (CCR1), 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used for the separation. Membranes were probed overnight at 4°C with anti-rat MIP1α polyclonal antibody (BioVision) diluted at 1:100 or anti-CCR1 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:100. The blots were washed and incubated for 1 h with biotinylated anti-species-specific secondary antibodies (Amersham Biosciences, Piscataway, NJ) and then visualized using ECL Western Blotting Detection Reagent (Amersham Biosciences). To ensure equal protein loading, the blots were striped with Restore Western Blot Stripping Buffer (Pierce) and reprobed with anti-β actin antibody (dilution 1:2000; Sigma–Aldrich) for 1 h at room temperature.

For the detection of serum MIP1α, GRO and IL-6, aliquots of 150 μg of protein from the sera of rats treated with $TiO_2$ for 16 weeks were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Anti-human GRO polyclonal antibody (BioVision) and anti-mouse IL-6 polyclonal antibody (Santa Cruz) were diluted 1:100. For detection of activated extracellular signal-regulated kinase (ERK) 1/2 and total ERK1/2, phospho ERK1/2 antiboby (Cell signaling Technology, Beverly, MA) and ERK1/2 antibody (Upstate, Lake placid, NY) were diluted 1:2000 and 1:25 000, respectively. The conditioned medium from alveolar macrophages, prepared as described above, was also subjected to western blot assay for MIP1α detection as described above. The blots were striped with Restore Western Blot Stripping Buffer (Pierce) and stained with Ponceau S solution (Sigma–Aldrich) for 10 min. The major band at 66 kDa was judged to be albumin and used as an internal control.

*In vitro cell proliferation assay*

A549 cells, a human lung cancer cell line, and the rat mammary cancer cell line C3 (19), derived from the H*ras*128 transgenic rats, were used in the *in vitro* cell proliferation assays. A549 or C3 cells were seeded into 96-well culture plates at $5 \times 10^3$ cells per well in 2% fetal bovine serum Dulbecco's modified Eagle's medium (Wako Chemicals Co., Ltd). After overnight incubation, the cells were treated as noted below, incubated for 72 h and the relative cell number was then determined.



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To investigate the effect of culture supernatant from alveolar macrophages on A549 cell proliferation, their media were replaced with diluted conditioned medium, and the cells were incubated for 72 h with 0, 5, 10 and 20 μg/ml of anti-MIP1α neutralizing antibody (R&D Systems, Minneapolis, MN) or with 20 μg/ml of irrelevant IgG. To investigate the effect of recombinant cytokines on A549 cell proliferation, 10, 50 or 100 ng/ml of recombinant protein, rat MIP1α (R&D Systems), human GRO (R&D Systems) or human IL-6 (R&D Systems), was added to A549 cells. To investigate the role of ERK in MIP1α-stimulated cell proliferation, A549 cells, treated with or without $2 \times 10^{-7}$ M of the specific MAPK/ERK kinase1 (MEK1) inhibitor PD98059 (Cell Signaling Technology) for 10 min, were treated with 50 ng/ml of MIP1α protein. To investigate the effect of reactive oxygen species (ROS) on cell proliferation, A549 cells, with or without pretreatment with 1 mM *N*-acetyl cysteine (Wako Chemicals Co. Ltd) for 30 min, were treated with 0.5 mM $H_2O_2$ (Wako Chemicals Co. Ltd). To investigate of the effects of MIP1α on rat mammary cells, C3 cells were treated with serially diluted recombinant rat MIP1α (0, 0.4, 2.0, 10 and 50 ng/ml, respectively; R&D Systems). For detecting the direct effect of $TiO_2$ particles on A549 and C3 cell proliferation, $5 \times 10^3$ A549 or C3 cells were cultured overnight and then treated with 10 or 50 μg/ml of $TiO_2$ particles.

After 72 h incubation, the relative cell number of A549 and C3 was determined using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) according to the manufacturer's instruction.

*Statistical analysis*

For *in vivo* data, statistical analysis was performed using the Kruskal–Wallis and Bonferroni–Dunn's multiple comparison tests. *In vitro* data are presented as means ± standard deviations. The statistical significance of *in vitro* findings was analyzed using a two-tailed Student's *t*-test and Bonferroni–Dunn's multiple comparison tests. A value of $P < 0.05$ was considered significant. The Spearman's rank correlation test was used to determine the association between $TiO_2$ dose and $TiO_2$ carcinogenic activity.

## Results

*Promoting effects of $TiO_2$ particles in DHPN-induced lung and mammary carcinogenesis*

Prior to initiation of the IPS-initiation–promotion and IPS 9 day studies, we conducted a preliminary study to confirm whether IPS would be a good tool to deliver $TiO_2$ particles to the alveoli. Rats were treated by IPS with India ink. We observed that ink particles of ~50 to 500 μm in diameter were diffusely distributed throughout the alveoli space (data not shown), confirming that IPS could deliver $TiO_2$ particles to the alveoli.

Four groups of female H*ras*128 rats were treated with +/− DHPN to initiate carcinogenesis and then treated with $TiO_2$ by IPS for 12 weeks: Group 1, DHPN alone; Group 2, DHPN followed by 250 μg/ml $TiO_2$; Group 3, DHPN followed by 500 μg/ml $TiO_2$ and Group 4, 500 μg/ml $TiO_2$ without DHPN. Microscopic observation in the lung showed scattered inflammatory foci, alveolar cell hyperplasia (Figure 1A) and adenomas in the DHPN-treated rats. The multiplicity (numbers per square centimeter lung) of hyperplasias and adenomas in Group 3 (DHPN followed by 500 μg/ml $TiO_2$) were significantly increased compared with Group 1 (DHPN followed by saline, Table I), and the increase showed a dose-dependent correlation (rho = 0.630, $P = 0.001$ for hyperplasias and rho = 0.592, $P = 0.029$ for adenomas) by the Spearman's rank correlation test. In the mammary gland, $TiO_2$ treatment significantly increased the multiplicity of adenocarcinomas (Figure 1C) and tended to increase the weight of the mammary tumors (Figure 1C). In the rats, which received $TiO_2$ treatment without prior DHPN treatment, alveolar proliferative lesions were not observed although slight inflammatory lesions were observed.

$TiO_2$ was distributed primarily to the lung, but minor amounts of $TiO_2$ were also found in other organs (supplementary Figure 1A is available at *Carcinogenesis* Online).

Various sizes of $TiO_2$ aggregates were observed in alveolar macrophages (Figure 1B). The $TiO_2$-laden macrophages were evenly scattered throughout the lung alveoli. The number of hyperplasias with $TiO_2$-laden macrophages was dose dependently increased (supplementary Table 1 is available at *Carcinogenesis* Online). This result suggests that $TiO_2$-laden macrophages may be involved in the promotion of alveolar hyperplasia.

The size distribution of $TiO_2$ particle aggregate is shown in Figure 1D. Of 452 particle aggregates examined, 362 (80.1%) were nanosize, i.e. <100 nm. Overall, the average size was 84.9 nm and the median size was 44.4 nm.

*IPS 9 day study—analysis of $TiO_2$*

Female SD rats were treated with $TiO_2$ by IPS over a 9 day period (Figure 2A). Microscopic observation showed scattered inflammatory lesions with infiltration of numerous macrophages mixed with a few neutrophils and lymphocytes in $TiO_2$-treated animals. Overall, the number of macrophages in the alveoli was significantly increased in the $TiO_2$-treated animals (Figure 2B). As expected from the results of the IPS-initiation–promotion study, alveolar proliferative lesions were not observed (Figure 2C).

Morphologically, $TiO_2$ particles were observed as yellowish, polygonal bodies in the cytoplasm of cells (Figure 2D). These cells are morphologically distinct from neutrophils and strongly positive for CD68 (Figure 2E), indicating that the $TiO_2$ engulfing cells were macrophages. $TiO_2$ aggregates of various sizes were found in macrophages, and aggregates larger than a single macrophage were surrounded by multiple macrophages (supplementary Figure 1B is available at *Carcinogenesis* Online)

TEM also showed electron dense bodies in the cytoplasm of macrophages (Figure 2F and G). These bodies were found exclusively in macrophages and not found in the alveolar parenchyma, including alveolar epithelium and alveolar wall cells, or in any other cell type. The shape of the electron dense $TiO_2$ particles in the cytoplasm was quite similar to that observed in preparations taken from $TiO_2$ suspensions before administration (Figure 2H and supplementary Figure 1C and D is available at *Carcinogenesis* Online). Individual $TiO_2$ particles were rod-like in shape (supplementary Figure 1C is available at *Carcinogenesis* Online).

Element analysis by TEM and X-ray microanalysis indicated that these electron dense bodies were composed primarily of titanium particles (supplementary Figure 1E and F-1 and 1F-2 is available at *Carcinogenesis* Online). Titanium was not observed in the surrounding alveolar cells without electron dense bodies (supplementary Figure 1F-3 is available at *Carcinogenesis* Online). The size distribution of $TiO_2$ particle aggregates is shown in Figure 2I. Of 2571 particle aggregates examined, 1970 (76.6%) were <100 nm and five particles were >4000 nm in size. Overall, the average size was 107.4 nm and the median size was 48.1 nm.

*IPS 9 day study—analysis of oxidative stress and inflammation-related factors in the lungs of wild-type rats*

IPS of $TiO_2$ particles significantly increased SOD activity (Figure 3A) and 8-OHdG levels (Figure 3B) in the lungs of wild-type rats, but not in the mammary glands. Analysis of the expression levels of 12 cytokines using suspension array indicated that administration of $TiO_2$ particles significantly upregulated the expression of MIP1α, GRO and IL-6 in the lung tissue of wild-type rats (supplementary Table 2 is available at *Carcinogenesis* Online). MIP1α levels were slightly elevated (0.4 pg/mg protein) in the mammary gland (Figure 3C), although the elevation was not statistically significant. Elevation of MIP1α in the lung tissue of animals treated with $TiO_2$ particles was confirmed by western blotting (Figure 3D).

Immunohistochemically, MIP1α was detected in the cytoplasm of alveolar macrophages with phagocytosed $TiO_2$ particles (Figure 3E upper, stained in red) and these macrophages could be found in hyperplastic lesions of the lung (supplementary Figure 2A and B is available at *Carcinogenesis* Online). MIP1α was not detected in macrophages without $TiO_2$ particles (Figure 3E lower). Expression of CCR1, the major receptor of MIP1α, was observed in the lung; IPS of $TiO_2$ particles had little or no effect on CCR1 expression (supplementary Figure 2C is available at *Carcinogenesis* Online).

*Effect of MIP1α on proliferation of a human lung cancer cell line in vitro*

Alveolar macrophages were isolated from the lungs of SD rats and were confirmed to be macrophages by morphology and CD68 staining



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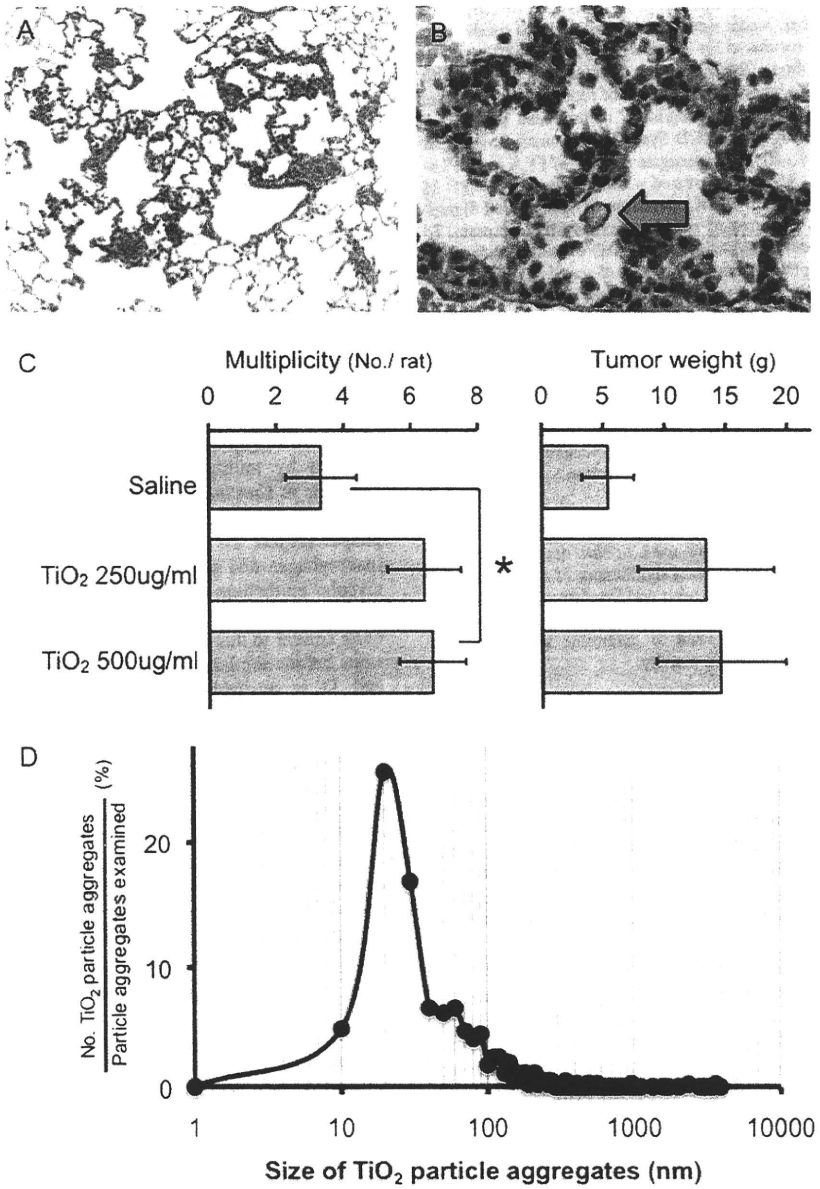


**Fig. 1.** Promoting effects of $TiO_2$ particles in DHPN-induced lung and mammary carcinogenesis (**A**) Alveolar hyperplasias observed in the lung of an H*ras*128 rat receiving DHPN and 500 μg/ml $TiO_2$ particles. (**B**) Alveolar macrophages with $TiO_2$ particles were also observed in hyperplasia lesions. (**C**) IPS of $TiO_2$ particles significantly increased the multiplicity of adenocarcinomas in the mammary gland and tended to increase the size of mammary tumors. (**D**) The size distribution of $TiO_2$ particle aggregates; among 452 particle aggregates examined, 362 (80.1%) were nanosize, i.e. <100 nm in diameter.

(data not shown). The macrophages were treated with $TiO_2$ particles suspended in saline (Figure 4A). $TiO_2$ induced secretion of MIP1α into the culture media (Figure 4B), and the culture medium collected from macrophages treated with $TiO_2$ particles promoted proliferation of A549 cells, whereas culture media collected from unexposed macrophages did not (Figure 4C). MIP1α neutralizing antibodies attenuated the promotion of A549 proliferation in a dose-dependent manner (Figure 4C). MIP1α-induced cell proliferation was also significantly suppressed by the ERK inhibitor PD98059 (Figure 4D). In addition, MIP1α increased ERK phosphorylation and PD98059 diminished ERK phosphorylation (Figure 4E).

We also examined the effect of MIP1α, GRO and IL-6. $H_2O_2$ and $TiO_2$ on the proliferation of A549 cells. MIP1α increased cell proliferation in a dose-dependent fashion, but GRO and IL-6 did not (supplementary Figure 3A–C is available at *Carcinogenesis* Online). $H_2O_2$ significantly suppressed cell proliferation, and antioxidant treatment diminished this suppression. Antioxidant treatment did not affect MIP1α-induced cell proliferation (supplementary Figure 3D is available at *Carcinogenesis* Online). These results suggest that ROS have no effect on tumor cell growth in this experiment.

In addition, $TiO_2$ did not directly increase proliferation of A549 cell (supplementary Figure 3E is available at *Carcinogenesis* Online).

*Mechanism analysis of the promotion of mammary carcinogenesis*

MIP1α was markedly elevated in the serum of the H*ras*128 rats treated with $TiO_2$ particles (Figure 5A). Serum levels of IL-6 were not changed by $TiO_2$ treatment and GRO was not detected in the serum



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**Table I.** Effect of TiO2 on incidence and multiplicity of DHPN-induced alveolar hyperplasia and adenoma of the lung

| Treatment | No. of rats | Alveolar hyperplasia | | Lung adenoma | |
|---|---|---|---|---|---|
| | | Incidence (%) | Multiplicity ## (no./cm$^2$) | Incidence (%) | Multiplicity # (no./cm$^2$) |
| Saline | 9 | 9 (100) | 5.91 ± 1.19 | 0 | 0 |
| n$TiO_2$ 250 mg/ml | 10 | 10 (100) | 7.36 ± 0.97* | 1 (10) | 0.10 ± 0.10 |
| n$TiO_2$ 500 mg/ml | 11 | 11 (100) | 11.05 ± 0.87** | 4 (36) | 0.46 ± 0.21* |

*$P < 0.05$, **$P < 0.001$ versus saline control.
#$P < 0.05$, ##$P < 0.001$ in trend test (Spearman's rank correlation test).

(Figure 5A). MIP1α was slightly elevated in the mammary glands of these animals (Figure 5B); possibly, the elevated MIP1α detected in the mammary tissue was due to contamination by MIP1α in the serum. Recombinant MIP1α promoted the proliferation of C3 cells in a dose-dependent manner; a slight induction could be seen at a dose of 400 pg/ml and became statistically significant at the dose of 50 ng/ml (Figure 5C). Expression of CCR1, the major receptor of MIP1α, was observed in mammary tissue, and as in the lung, IPS of $TiO_2$ particles had little or no effect on CCR1 expression (data not shown). $TiO_2$ did not directly increase proliferation of C3 cells (supplementary Figure 3F is available at *Carcinogenesis* Online).

## Discussion

To elucidate the mechanism underlying rat lung carcinogenesis by $TiO_2$ inhalation, we chose IPS. Although this method may be less physiological than the aerosol inhalation system, we observed that agglomerates and aggregates of $TiO_2$ particles from nano to micro size (mean diameter 107.4 nm) were diffusely distributed throughout the lung including peripheral alveoli, and they did not cause obstruction of the terminal bronchioles. Accordingly, IPS of $TiO_2$ particles can be expected to act similarly to aerosol inhalation of $TiO_2$.

Occupational exposure limits for $TiO_2$ in 13 countries or regions are 5–20 mg/m$^3$ (20), which results in $TiO_2$ exposure limits of 0.27–1.07 mg/kg body wt/day; calculations based on the human respiratory volume. In the present study, a total of 1.75 mg was administered per rat for 12 weeks in the high-dose group, resulting in a dose of 0.104 mg/kg body wt/day. Therefore, the dose we used in the present study was lower than the occupational exposure limit.

$TiO_2$, nanoscale and larger sized is evaluated as a Group 2B carcinogen by World Health Organization/International Agency for Research on Cancer (4) based on 2 year animal aerosol inhalation studies (3). We conducted the present carcinogenesis study using a two-step initiation–promotion protocol as a surrogate for a 2 year long-term protocol. Our study demonstrated that $TiO_2$ particles increased the multiplicity of alveolar cell hyperplasia and adenoma in the two-step IPS-initiation–promotion protocol. We used these lesions as endpoints in carcinogenicity testing because chemically induced tumors appear to be derived from hyperplasic lesions that progress to adenoma and carcinoma (21).

Several bioassay protocols based on the two-step carcinogenesis theory have been developed as practical and sensitive assays, and the compounds that exhibit promotion activity are considered to be carcinogens (22–29). Thus, our experimental design may be a practical surrogate for the long-term lung carcinogenesis protocol.

It should be noted that proliferative lesions including alveolar cell hyperplasia and adenomas were not found in the groups subjected to $TiO_2$ particle administration without prior treatment of DHPN. This is due to the weak carcinogenic potential and short duration of exposure to $TiO_2$ particles. Using the two-step IPS-initiation–promotion protocol, however, we did observe carcinogenic activity by this weak carcinogen. Thus, the two-step IPS-initiation–promotion protocol is an appropriate system to study carcinogenesis of $TiO_2$ particles and approximates long-term $TiO_2$ inhalation studies (3,11).

We next conducted a mechanism analysis of $TiO_2$ particle carcinogenesis focusing on the initial events induced by exposure to $TiO_2$ particles. Treatment with $TiO_2$ resulted in a modest infiltration of inflammatory cells into the alveolar space and septal wall, but the primary effect was a marked increase in the number of macrophages in the alveoli, and many of these macrophages contained phagocytosed $TiO_2$ particles. Alveolar macrophages play an important role in deposition and clearance of mineral fibers/particles, and macrophage activity is known to be strongly associated with inflammatory reactions and carcinogenesis caused by fibers and particles in the lung, including asbestos (30–33). ROS are known to be produced by macrophages upon particle phagocytosis (34,35). Clinical and experimental studies indicate that ROS production and resultant oxidative stress play an important role in cellular and tissue damage, inflammation and fibrosis in the lung. In our study, a significant increase in the activity of SOD and 8-OHdG formation in the lung were observed, indicating increased ROS production and DNA damage. Because macrophages are unable to detoxify $TiO_2$ particles, the reaction against these particles would be continuous over an extended period of time. This condition is associated with high levels of ROS production (36) and tissue toxicity (37).

Cytokine analysis of the lung tissue indicated that among the 12 cytokines examined, expression of IL-6, GRO and MIP1α were significantly higher in the $TiO_2$-treated group than in the vehicle group (supplementary Table 1 is available at *Carcinogenesis* Online). IL-6 is a pro-inflammatory cytokine that is involved in host defense as well as cancer development (38,39). IL-6 has been shown to be increased in lung tumor tissue (40,41) and in the sera of lung cancer patients (42). GRO, a member of the CXC chemokine family, has been shown to be involved in inflammatory responses, chemoattraction (43), carcinogenesis (44,45) and tumor progression (46). Thus, IL-6 and GRO may be involved in the promotion of lung carcinogenesis by $TiO_2$ (47).

Of the three cytokines induced by exposure to $TiO_2$ particles, however, we were particularly interested in MIP1α. This cytokine was not only induced in the lung tissue of $TiO_2$-treated rats, but, unlike IL-6 and GRO, it was also found in the serum of these animals. MIP1α is a member of the CC chemokine family and is primarily associated with cell adhesion and migration (17), proliferation and survival of myeloma cells (48). It is produced by macrophages in response to a variety of mineral particle-induced inflammatory stimuli (18). Our results indicate that expression of MIP1α by alveolar macrophages enhances the proliferation of A549 cells. Expression of CCR1, the major receptor of MIP1α, was observed in the lung tissue, rendering lung cells receptive to MIP1α induction of proliferation. Lung damage and inflammation induced by $TiO_2$ particles has also been reported to be associated with increased cell proliferation of lung epithelium cells (49), which is consistent with our results.

The MEK1–ERK-signaling pathway has been shown to be involved in CCR1 signaling (48). In the present study, the MEK1-specific inhibitor PD98059 suppressed MIP1α-induced cell proliferation and ERK phosphorylation. These results suggest that MEK1 is one of the downstream signaling molecules of MIP1α and the MEK1–ERK-signaling pathway may be partially involved in MIP1α signaling.

It should be noted that, in our IPS-initiation–promotion protocol, $TiO_2$ exposure also promoted DHPN-initiated mammary carcinogenesis. Our results suggest that MIP1α secreted by alveolar macrophages and transported via the circulatory system caused



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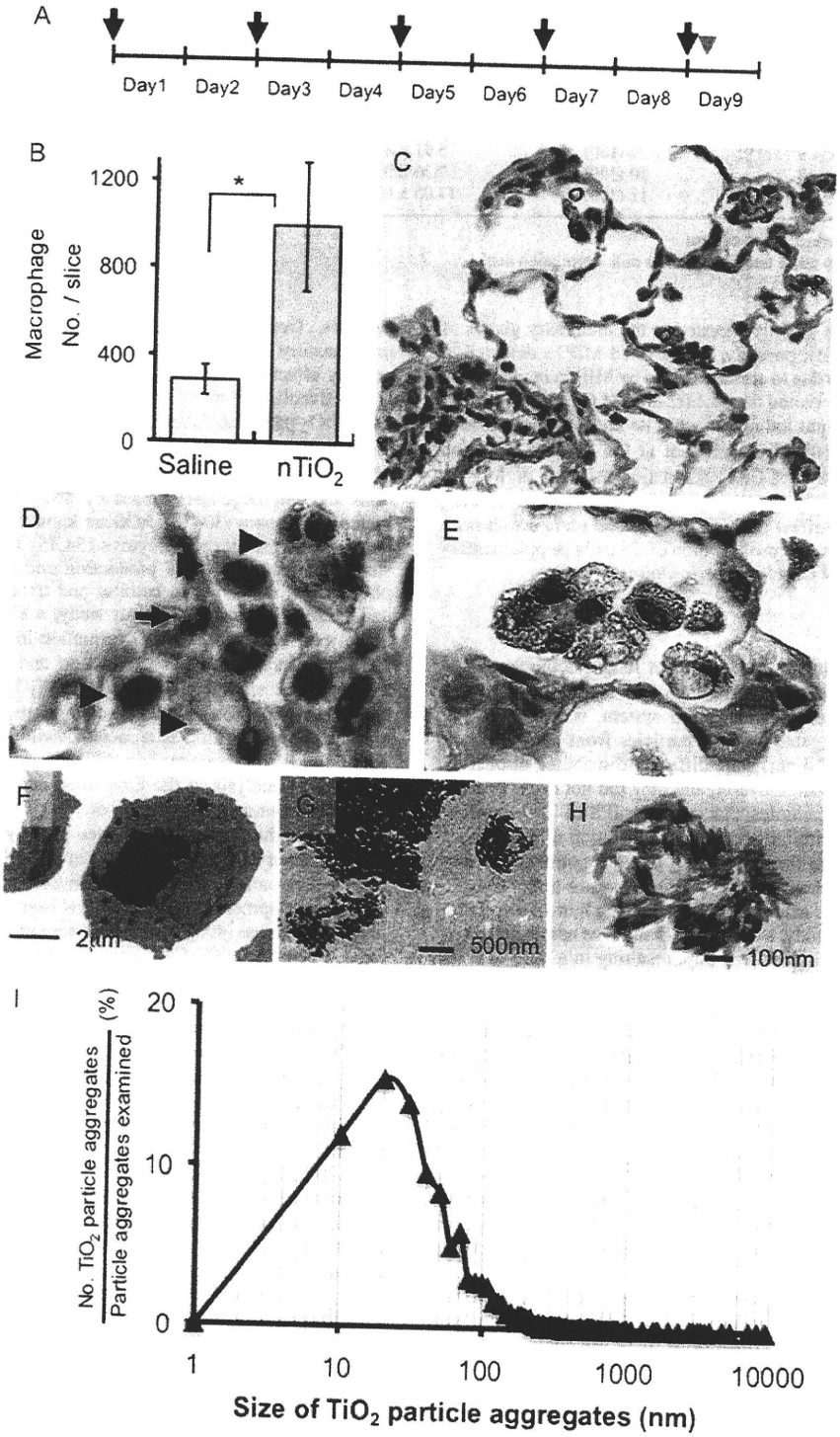


**Fig. 2.** $TiO_2$ particles in alveolar macrophages by light and electron microscopy (**A**) Twenty female SD rats (wild-type counterpart of H*ras*128) aged 10 weeks were treated by IPS with 0.5 ml suspension of 500 μg/ml $TiO_2$ particles in saline five times over a 9 day period. Arrows and arrowhead indicates IPS treatment and killing of the animals, respectively. (**B**) IPS of $TiO_2$ particles significantly increased the number of macrophages in the alveoli. (**C**) Inflammatory reactions were observed in the lung with slight infiltration of macrophages, neutrophils and lymphocytes. (**D**) $TiO_2$ particles were observed in alveolar macrophages (hematoxylin and eosin staining). Arrowheads indicate macrophages and the smaller cell indicated by the arrow is a neutrophil with its characteristic multilobular nucleus. (**E**) The multinucleated cells containing these particles were positive for the macrophage marker CD68 (Alkaline phosphatase reaction, red color). (**F**) TEM findings showed that $TiO_2$ particles of various sizes (~50 nm to 5 μm) were observed phagocytosed by alveolar macrophages. (**G**) Electron dense bodies were aggregates of $TiO_2$ particles. (**H**) TEM findings of $TiO_2$ particles in saline suspension before IPS. The shape of the $TiO_2$ particle aggregates was similar to those observed in macrophages. (**I**) The size distribution of $TiO_2$ particle aggregates: of 2571 particle aggregates examined, 1970 (77.1%) were <100 nm. The average size was 107.4 nm and the median size was 48.1 nm.



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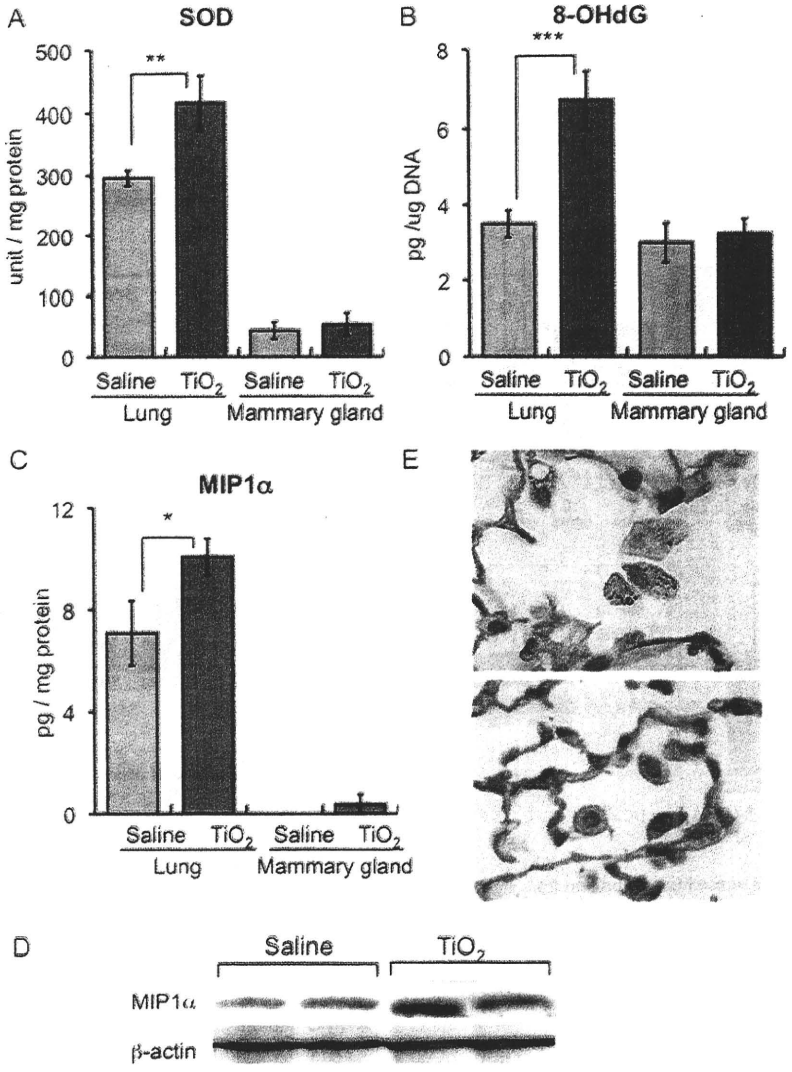


**Fig. 3.** Inflammatory factors upregulated in the lungs of wild-type rats by IT-spraying of $TiO_2$ particles in the IPS 9 day study (**A**) SOD activity and (**B**) 8-OHdG level in wild-type rats treated with $TiO_2$ particles or saline. (**C**) MIP1α protein level was significantly increased (142%) in the lung tissue of wild-type rats treated with $TiO_2$ (suspension array analysis). MIP1α was detected in the mammary gland of the $TiO_2$ group but not in the vehicle group. (**D**) In western blotting, expression of MIP1α was increased in the $TiO_2$ group compared with vehicle group. (**E**) MIP1α was immunohistochemically detected in alveolar macrophages containing $TiO_2$ particles (upper) but was not detected in macrophages of rats that were not exposed to $TiO_2$ particles (lower).

proliferation of mammary epithelial cells and thereby promoted mammary carcinogenesis. As with the lung, CCR1 was expressed by mammary cells, rendering these cells receptive to MIP1α induction of proliferation. While MIP1α secreted by alveolar macrophages would be diluted by the blood volume and while these levels may not be high enough to increase mammary cell proliferation in a short *in vitro* proliferation assay, it is possible that continuous low level stimulation over the course of 12 weeks could increase mammary cell proliferation in the environment of the mammary gland *in vivo*. Another possibility is that $TiO_2$ particles may act directly on the mammary gland after translocation to the mammary gland from the lung. However, $TiO_2$ exposure of mammary carcinoma cells did not induce proliferation *in vitro*. It must be understood that promotion of DHPN-induced mammary carcinogenesis by $TiO_2$ particles was observed in *Hras*128 female rats, and these animals are very highly susceptible to mammary carcinogenesis (50). Although, the effects we observed on promotion of mammary carcinogenesis in these animals may not be directly relevant to most humans, people at high risk for mammary carcinogenesis, such as individuals harboring BRCA mutations, may be a relevant population as regards the risk presented by nanoscale $TiO_2$.

Although our observations are based on results obtained with a mixed population of nanoscale and larger sized particle aggregates, size analysis indicated that 80.1% of them were nanoscale (<100 nm in diameter) in the 16 week IPS-initiation–promotion study and 76.6% were nanoscale in the IPS 9 day study. Thus, the results can be interpreted as being strongly associated with nanoscale particle aggregates.

In conclusion, the IPS-initiation–promotion protocol detected $TiO_2$ carcinogenic activity in the rat lung and is therefore comparable, at least for $TiO_2$ inhalation, to a long-term whole body inhalation carcinogenesis study. We also elucidated a plausible mechanism for the carcinogenic effect of $TiO_2$ particles in the rat lung. Phagocytosis of $TiO_2$ particles by alveolar macrophages resulted in ROS production and DNA damage and increased expression of MIP1α. MIP1α in turn was able to enhance proliferation of lung epithelium cells. Thus, lung



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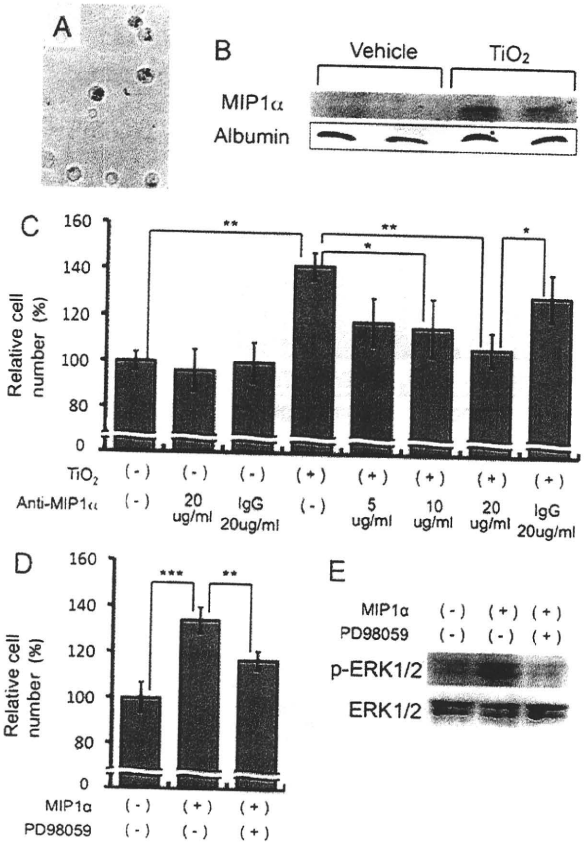


**Fig. 4.** Growth stimulation effects of conditioned medium from alveolar macrophages on human lung cancer cell lines. (**A**) Primary cultured alveolar macrophages of rats were treated with $TiO_2$ particles. (**B**) MIP1α was detected in the culture medium. (**C**) The number of A549 cells was significantly increased by addition of conditioned medium from alveolar macrophages treated with $TiO_2$ particles. MIP1α neutralizing antibody attenuated this effect in a dose-dependent manner. Irrelevant IgG was used as control antibody. (**D**) MIP1α-induced cell proliferation was significantly suppressed by the ERK inhibitor PD98059. (**E**) MIP1α increased ERK phosphorylation and PD98059 diminished this phosphorylation.

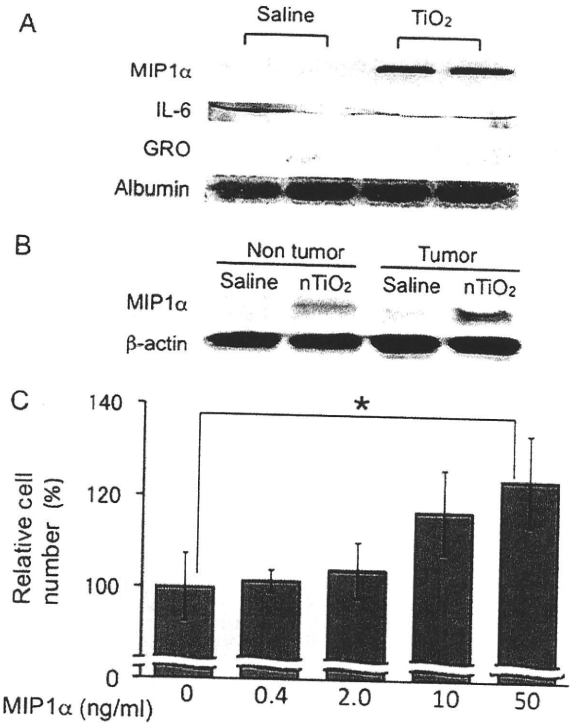


**Fig. 5.** Promotion effects of MIP1α on proliferation of a rat mammary cancer cell line, C3 (**A**) MIP1α was detected in the serum of the H*ras*128 rats treated with $TiO_2$ but not in vehicle control rats in the 16 week study. No difference in IL-6 in the serum was observed and GRO was not detected in the serum. (**B**) MIP1α levels are slightly elevated in non-tumor and tumor tissue of the mammary gland of animals treated by IPS with $TiO_2$ particles in the 16 week study. (**C**) Recombinant MIP1α increased the number of rat mammary carcinoma C3 cells in a dose-dependent manner ($P = 0.0127$).

tissue exposed to $TiO_2$ particles exhibits increase in both DNA damage and proliferation. Importantly, a similar mechanism would function in humans in the promotion of lung carcinogenesis associated with inhalation of $TiO_2$ particles and other nanoparticles with the capacity to form aggregates. In addition, $TiO_2$ administered to the lung had carcinogenic activity in the H*ras*128 transgenic rat mammary gland; this carcinogenic activity is probably mediated via serum MIP1α resulting from expression of MIP1α by alveolar macrophages. This finding may indicate that exposure of $TiO_2$ particles is a risk factor for mammary carcinogenesis in predisposed populations, such as individuals with BRCA mutations.

## Supplementary material

Supplementary Figures 1–3 and Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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SYMPOSIUM PRESENTATION

# Risk Assessment Studies of Nanomaterials in Japan and Other Countries

**Hiroyuki Tsuda**

## Abstract

**Recent developments in nanoparticle (NP) technology and their commercial production have raised concern regarding NP risk to health and the environment. The toxicological characteristics of NP may not be similar to that observed in pre-NP materials because of the enormous differences in size and surface area. Thus, careful risk evaluation studies are required. Since some NP have already been produced and introduced into the market, before a suitable framework enabling risk management has been firmly established, toxicological studies based on the specificity of NP which are not subordinate to their commercial production are indispensable. The summary of nanotoxicology studies shown below clearly indicates that compared with the UK, EU, USA, and other countries, Japanese studies regarding metals and SWCNT are far from sufficient to evaluate risk.**

**Key Words:** Nanoparticles - toxicology - carcinogenicity - titanium dioxide - carbon black

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## Introduction

The safety of our living environment can be secured by the balanced function of three elements: risk assessment, risk management and risk communication. The first of these elements, risk assessment, must be addressed first, since without reliable risk assessment, risk communication and risk management can not function. Importantly, for reliable risk assessment long-term animal studies are indispensable.

These principles, of course, hold true for engineered nanoparticles. Unfortunately, the risk assessment data for engineered nanoparticles are rather fragmentary. However, the available findings do present a disturbing picture of potential carcinogens entering the market place. Engineered nanoparticles included in this review include nano-size titanium dioxide ($nTiO_2$), carbon black (nCB), single-walled carbon nanotubes (SWCNT), multiple-walled carbon nanotubes (MWCNT) and fullerenes (C60). A summary of the testing so far performed shows that data are limited and Japan is not putting the priority this subject deserves (see Table 1) and Tsuda et al (2009), for a review.

**Table 1. Number of Subacute/Chronic Toxicity Tests (PubMed, ~2007)**

| | ● | ■ | ▤ | Total |
|---|---|---|---|---|
| $TiO_2$ | 1 | 2 | 2[a] | 5 |
| S/MW-CNT | 2 | | | 2 |
| Fullerenes | | 1 | | 1 |
| Uf-Carbon black | | | 4[a] | 4 |
| Total | 0 | 3 | 6 | 9 |

[a]Carcinogenic

## Overall Evaluation and Proposal for the Future

During the development and marketing of nanomaterials, risk assessment of these new products has been perfunctory at best. While nanomaterials have undeniable benefits, their use also has undeniable potential risk. This risk must be addressed in an unbiased and thorough manner. Only after the toxicity of the various nanomaterials is understood can their true benefits be realized.

In rodent studies, $nTiO_2$ whether administered by inhalation or intratracheal instillation was shown to induce lung tumors with characteristic squamous cell morphology in female rats. These nanomaterials did not induce lung tumors in male rats. Our own studies have also shown that instillation of $nTiO_2$ into the lungs of female rats showed tumor promoting activity and resulted in elevated ROS-mediated damage and production of inflammatory cytokines. It is reasonable to assume that other metal-derived nanoparticles, such as aluminium and copper nanoparticles, and metal containing nanoparticles, for example nCB-metal mixtures and SWCNT and MWCNT preparations, are also capable of producing these effects.

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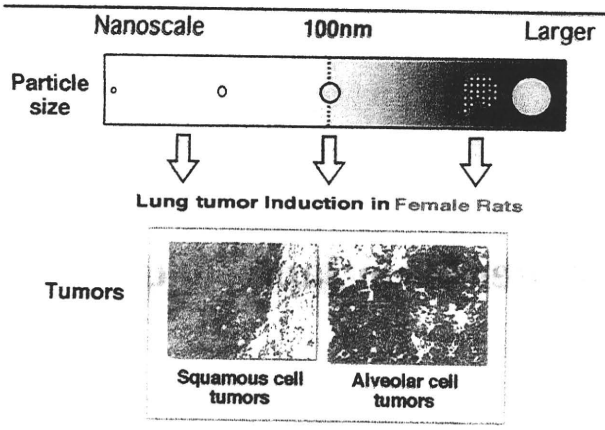


**Figure 1. Schematic Presentation of Carcinogenic Effects of $TiO_2$, Carbon Black.** Carcinogenic effects were elicited by both nano-scale and larger sized particles

Nanoparticles such as $nTiO_2$, nCB, SWCNT and MWCNT when intratracheally administered, were detected by light microscope as aggregates or agglomerates and these forms are reported to induce foreign body granulation tissue with various degree of inflammatory reaction. Although the relevance of foreign body-induced chronic inflammation to carcinogenesis is not clearly established, it is possible that reactive oxygen species (ROS) produced by macrophages attempting to destroy the foreign material in the inflammation site may cause DNA damage associated with carcinogenesis. Another possible contributing factor is metal, for example from metal-derived nanoparticles such as $TiO_2$ or from metal contaminants: these metals could also be involved in ROS production. Thus, it is possible that the observed carcinogenic effect is not specific to nanoparticles but rather associated with their ability to induce persistent foreign body-induced chronic inflammation and/or introduce metals into susceptible sites. For example, $TiO_2$ and carbon blacks larger than 100nm in diameter are known to induce lung tumors including similar squamous cell morphology (Nikula, 2000); (Pott and Roller, 2005) and both of these materials (larger than nano size) are classified as into group 2B (possibly carcinogenic to humans) by WHO/IARC (see Figure 1).

Mechanisms for mesothelioma induction by MWCNT in mice and rats have not been elucidated yet. A possible contributing factor is metal: Transition metals, such as iron, are commonly used as a catalytic center in the formation of CNTs, and contaminating metal in SWCNT and MWCNT particles could catalyze the formation of ROS by the Fenton reaction (Liu and Okada, 1994). One example of this type of toxicity is that human keratinocytes exposed to SWCNT were killed by ROS in the media (Shvedova et al., 2003). Another possible contributing factor is the length of the MWCNT (Pott and Roller, 2005; Muller et al., 2009; Sakamoto et al., 2009).

As noted at the beginning of this review, for reliable risk assessment long-term animal studies are indispensable. This is particularly true for risk assessment of potential carcinogens. The standard for the evaluation of the carcinogenic potential of a test chemical is testing in two rodent species, generally rats and mice, of each sex, at 3 doses (0, low and high) of the test chemical for up to two years. In the studies conducted to date concerning the carcinogenic risk presented by nanoparticles, there is a noticeable lack of long term testing: No long-term tests of any type have been reported for either SWCNT or fullerenes. Importantly, the primary goal of risk assessment is not to simply ban a product from the market place, but rather to determine product safety and establish guidelines lines for its production and use and promote consumer confidence. Given the known ability of many nanomaterials to induce mechanisms which are active in humans that are risk factors for carcinogenesis, for example ROS and inflammatory cytokine production, the continued introduction of these materials into the market is alarming. Establishing the safety of these materials is urgently needed.

In this short review, available in vivo data concerning the carcinogenic effects of $nTiO_2$, nCB, SWCNT and MWCNT, and Fullerenes is outlined. Of these, $nTiO_2$ and nCB are classified as possibly carcinogenic to humans. Testing of the carcinogenic activity of MWCNT produced mixed results. SWCNT and fullerenes have no carcinogenic activity in the studies conducted to date, however, toxicity testing of these materials has been quite limited and both of these materials have the potential to produce ROS. The observations noted here may apply to possible carcinogenic risk of other nanoparticles because of shared mechanisms of induction of inflammatory lesions and ROS generation. Our conclusions are that nanoparticles are clearly potentially toxic/carcinogenic to humans and their toxicity must be assessed, and their production and use managed appropriately.

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