— Research Letter —

Nanoscale Titanium Dioxide Particles Modulate Signaling Cascades for Tumor Necrosis Factor-α Release from Macrophages

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Toxicological and biomedical effects of nanoparticles to living subjects should be elucidated before use for safety. To address this point, we attended to proinflammatory cytokine release from macrophage-like RAW 264.7 cells as a measure of the impact of nanoscale particles to living subjects. We explored the specific impacts of TiO_2 particles on the signaling cascades for proinflammatory cytokine and tumor necrosis factor-α (TNF-α release using a bioluminescent coculture system and an ELISA assay in the presence of various chemotherapeutic agents or biochemical inhibitors. This evaluation revealed that the proinflammatory cytokine release from the RAW 264.7 cells is synergically enhanced by the mixture of TiO_2 particles and interferon-γ (IFN-γ), and TNF-α release is modulated by 40 nm TiO_2 particles via a mitgen activated protein kinase (MAPK)/nuclear factor-kappa B (NF-κB) pathway. The TNF-α release is also affected by the agonist of glucocorticoid receptor in both basal and TiO_2-particle-activated conditions. The present study evidences that nanoscale TiO_2 particles exert a modulator to the initial steps of inflammation, called TNF-α release from RAW 264.7 cells.

Key words —— Nanoscale titanium dioxide particle, tumor necrosis factor-α, inflammation, biochemical reagent

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INTRODUCTION

To date, few studies have examined toxicological effects of direct and indirect exposure of nanomaterials to living subjects; no clear guidelines exist to quantify these effects.[1] Therefore, a vital task of toxicological and biomedical studies of nanomaterials is to measure their potential effects on cell viability, immortalization, necrosis, inflammatory response, and apoptosis before using the materials in industrial and biomedical applications.

Recent studies have unveiled specific details of signaling pathways in the nuclear factor-kappa B (NF-κB) activation by nanoparticles inside mammalian cells via pattern recognition receptors (PRRs) such as class A scavenger receptors (SRAs) and Toll-like receptors (TLRs).[2] On the other hand, a receptor-unmediated signaling pathway for NF-κB activation, called redox-responsive signaling, has also been proposed.[3] According to that study, titanium particles interact with the cell membrane, causing lipid peroxidation and generating free radicals. The reactive oxygen species activate NF-κB expressing tumor necrosis factor-α (TNF-α): TNF-α is a famous proinflammatory cytokine, and which works as a key signaling transducer for inflammation.

We previously established a cocultured cell-based assay with macrophage-like RAW 264.7 and human cervical carcinoma-derived HeLa cells for scoring proinflammatory cytokine release, named "a cocultuer system."[4]

Herein, we demonstrate in detail the effects of nanoscale TiO_2 particles on the signaling pathway for the TNF-α release as a proinflammatory cytokine in RAW 264.7 cells using various chemotherapeutic agents and biochemical inhibitors.

MATERIALS AND METHODS

Reagents —— As materials, this study used 40-nm titanium oxide (IV) particles (TiO_2, a mixture of anatase and rutile; Sigma-Aldrich, St. Louis, MO, U.S.A.), a high sensitivity Western blot detection kit (ECL advance; Amersham Biosciences Corp., Sunnyvale, CA, U.S.A.), an enzyme-linked immunosorbent assay (ELISA) kit for mouse recombinant TNF-α (IBL, Gunma, Japan), and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophore-

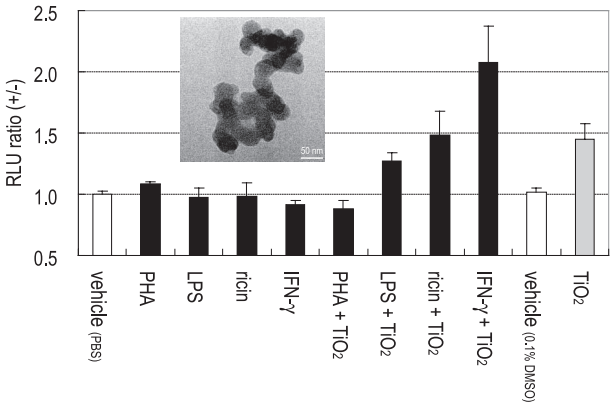

Fig. 1. Synergistic Effects of TiO_2 Particles on the Proinflammatory Cytokine Release from Macrophages in the Presence of a Biotherapeutic Agent

The effects were estimated using a coculture system that consisted of macrophage RAW 264.7 cells for sensing TiO_2 particles and HeLa cells carrying a pair of luminescent indicators. The white bars show luminescence intensities from the cells upon stimulated with a vehicle [0.02 M PBS or 0.1% dimethyl sulfoxide (DMSO)]. The gray bar shows the luminescence intensities from the cells upon stimulation with 40-nm TiO_2 particles alone (n = 3). Inset shows transmission electron microscopy (TEM) images of 40-nm TiO_2 particles in a phosphate buffered saline (PBS) buffer. The image visualizes the contacting surface area of TiO_2 particles to RAW 264.7 cells.

sis (PAGE) gels (TEFCO, Tokyo, Japan). The protein additives, phytohemagglutinin (PHA), ricin, interferon-γ (IFN-γ), and lipopolysaccharide (LPS), were obtained from Wako (Osaka, Japan). The following biochemical inhibitors were purchased from Sigma-Aldrich: monensin (mon), dansylcadaverine (dansyl), tyrphostin AG 126 (tAG), H7, wortmannin (wort), U0126, and corticosterone (corti).

Determination of the Impacts of TiO_2 Particles on the TNF-α Release Using a Coculture System —— The modulative impacts of TiO_2 particles on the TNF-α release were first examined using a coculture system, previously established by us[4] (Fig. 1). HeLa and RAW 264.7 cells, members of the coculture system, were prepared in different culture plates: HeLa cells cultured in a 12-well microplate (5×10^4 cells per well) were co-transfected with the previously developed pair of bioluminescent probes, pcRDn-NLS and pcDRc-p50,[4] and cultured in a cell incubator (37°C, 5% CO_2) for 12 hr. On the other hand, RAW 264.7 cells were raised in 10-cm culture plates, and harvested by trypsinization; an aliquot of the cell solution (5×10^4 cells) was mixed with one of the following chemotherapeutic agents: vehicle (phosphate buffered saline, PBS), 1 ng/ml PHA, 1 ng/ml LPS, 1 ng/ml ricin, 1 ng/ml IFN-γ, 1 ng/ml PHA plus 50 μg/ml TiO_2 particles (40 nm), 1 ng/ml LPS plus 50 μg/ml TiO_2 particles (40 nm), 1 ng/ml ricin plus 50 μg/ml TiO_2 particles (40 nm), 1 ng/ml IFN-γ plus 50 μg/ml TiO_2 particles (40 nm), vehicle [0.1% dimethyl sulfoxide (DMSO)], or 50 μg/ml TiO_2 particles (40 nm). The stimulated concentrations of the chemotherapeutic agents were referred from previous studies.[5,6] The doses and effects of the inhibitors on the target signaling cascades were well examined in previous studies.[6,7]

TiO_2-concentration dependency of TNF-α release (particle size: 40 nm) was already examined in the Western blot analysis using anti-TNF-α antibody in Fig. 2, Inset in the present study, and also in other references.[8] The mixtures were injected into each well of the 12-well plate, which contained cultures of HeLa cells carrying the bioluminescent probe. Three hours after their addition, the recovered luciferase activities were estimated using an *Renilla* luciferase (RLuc) substrate solution (Promega, Fitchburg, WI, U.S.A.) and a luminometer (Minilumat LB9506; Berthold, Bad Wildband, Germany).

The brief working mechanism of the coculture system is as follow: First, RAW 264.7 senses TiO_2 particles and immediately secretes proinflammatory cytokines, representatively TNF-α.[9] The cytokines diffuse to the nearby contacting sensor HeLa cells to stimulate the nuclear transport of NF-κB there. Thus, we demonstrate the total inflammatory activities of nanoparticles based on the extent of nuclear transport of NF-κB occurring in the sensor cell.

The direct stimulational effect of TiO_2 particles on the HeLa cells in the coculture system were examined, and found not to trigger elevation of the bioluminescence from HeLa cells (data not shown). It shows that TiO_2 particles selectively stimulate the macrophage RAW 264.7 cells, but not the HeLa cells.

ELISA-based Determination of TNF-α Release —— The amounts of TNF-α release from RAW 264.7 cells in response to TiO_2 particles were measured with a commercial ELISA assay kit (IBL, Fig. 2). The RAW 264.7 cells were cultured to 90% confluence in a 12-well plate (1×10^5/well). The culture medium on the plate was replaced with fresh medium supplemented with one of the following biochemical reagents: 20 μM monensin (pH gradient inhibitor), 0.5 μM wortmannin [phosphoinositide 3 kinase (PI3-K) inhibitor], 0.5 μM U0126 [mitogen activated protein kinase kinase (MAPKK) inhibitor], 20 μM H7 [protein kinase C (PKC) inhibitor], 100 μM tyrphostin AG 126 (protein tyrosine kinase inhibitor), 100 μM dansyl-

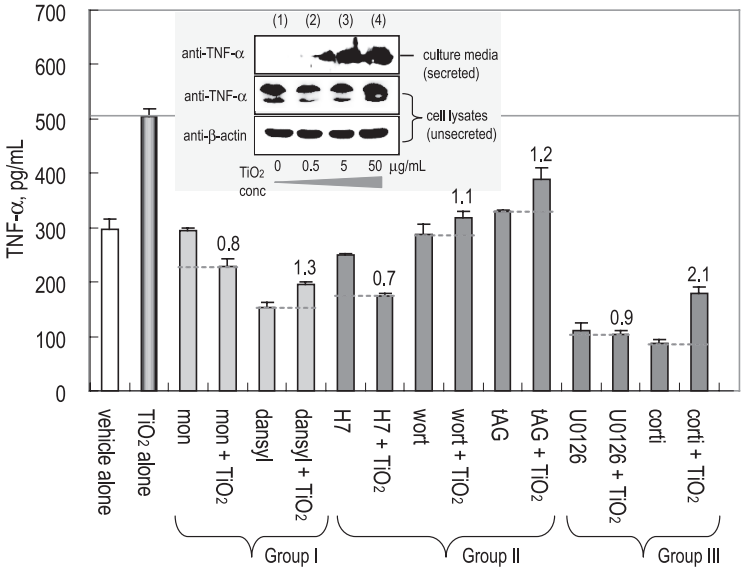

Fig. 2. An ELISA-based Determination of TNF-α Released into the Culture Media

The secreted amounts of TNF-α from RAW 264.7 cells were measured using an ELISA kit specific for TNF-α (n = 3). Abbreviations: dansyl, dansylcadaverine; mon, monensin; tAG, tAG 126; wort, wortmannin; corti, corticosterone. "TiO_2" on the X-axis exhibits the addition of 40-nm TiO_2 particles (final conc: 50 μg/ml). "Vehicle" means a stimulation of RAW 264.7 cells with the vehicle, PBS. The numbers on the bars shows ratios of the amounts of TNF-α release in the presence over absence of TiO_2 particles. Inset represents a Western blot analysis for determining the extent of TNF-α release from RAW 264.7 cells in response to 40-nm TiO_2 particles (final conc: 50 μg/ml). As a reference for the amounts of proteins that were electrophoresed, β-actin was stained with its specific antibody. Lane 1 shows blots of TNF-α from RAW 264.7 cells in the absence of TiO_2 particles, whereas lane 4 shows the blots of TNF-α in response to 50 μg/ml TiO_2 particles.

Table 1. List of Biochemical Reagents Used for this Study

"Dose" shows each final concentration of the biochemical reagents. The parenthesis shows the role name of each chemical.

Chemical name		Effects on cell signaling	Abbreviation	Dose
dansylcadaverine	(transamidase inhibitor)	Inhibit endocytosis	dansyl	100 μM
monensin	(pH gradient inhibitor)	Inhibit acidification	mon	20 μM
tyrphostin AG 126	(protein tyrosine kinase inhibitor)	Inhibit tyrosine phosphorylation	tAG	100 μM
U0126	(MAPKK inhibitor)	Inhibit MAPK phosphorylation	U0126	0.5 μM
H7	[protein kinase C (PKC) inhibitor]	Inhibit protein kinase C	H7	20 μM
wortmannin	(PI3 kinase inhibitor)	Inhibit PI3 phosphorylation	wort	0.5 μM
corticosterone	(stress hormone)	Inhibit inflammation	corti	1 μM

cadaverine (transamidase inhibitor), or 1 μM corticosterone (endogenous stress hormone). The inhibitor concentrations were referred from earlier papers.[6, 7, 10] The administered dose and expected inhibitory effects of the biochemical reagents inside cells are listed in Table 1. Ten minutes after pretreatment with the reagents, 40-nm TiO_2 particles were added to the wells to be 50 μg/ml. Three hours following incubation of the cells at 37°C, the TNF-α that was secreted from the RAW 264.7 cells into each culture medium was quantified using a 96-well ELISA assay kit according to the manufacturer's manual.

Western Blot Analysis —— The TNF-α secreted from RAW 264.7 cells in response to TiO_2 particles was determined using Western blot analysis (Fig. 2, Inset). The RAW 264.7 cells were cultured in 12-well plates, then stimulated with differing concentrations of 40-nm TiO_2 particles in the presence of IFN-γ. Four hours after stimulation, the upper culture medium and the RAW 264.7 cells on the well bottom were boiled respectively with lysis buffer (1% SDS, 10% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue, 50 mM Tris-HCl, pH 6.8) at 95°C for 5 min. The boiled samples were then electrophoresed in 15% acrylamide gel and transferred to a nitrocellulose membrane. The membrane was incubated with rat anti-TNF-α antibody or with mouse anti-β-actin antibody, and was incubated extensively with peroxidase-conjugated anti-

mouse IgG antibody. The blots were visualized using a chemiluminescence reagent (GE Healthcare, St. Giles, U.K.) and a luminescence image analyzer (LAS-1000; FujiFilm, Tokyo, Japan).

RESULTS AND DISCUSSION

Proinflammatory Cytokine Release as a Measure of the Impact of TiO_2 Particles

Toxicological and biomedical effects of direct and indirect exposure of nanoparticles to living subjects have been debated.[11] It should be explained whether nanoparticles can cause a synergetic, adverse effect on living subjects with biotherapeutic agents even though they alone might show weak toxicological effects. A detailed signaling pathway triggered by nanoparticles should be elucidated to explore their potential activities in the physiological circumstances of living subjects.

To address those points, we attended to TNF-α as an indicator proinflammatory cytokine, connecting nanoparticles with inflammation. Thus, we speculated that the levels of proinflammatory cytokine release are a potential measure of the impact of nanoscale TiO_2 particles to the signaling cascades for proinflammatory cytokine release from macrophage RAW 264.7 cells.

We first examined the synergistic impacts of TiO_2 particles with chemotherapeutic agents on the proinflammatory cytokine release from RAW 264.7 cells (Fig. 1), considering the biological and medical utilities of chemotherapeutic agents such as PHA and LPS. Second, the intracellular signaling pathways for TNF-α release from RAW 264.7 cells in response to TiO_2 particles were explored in detail using biochemical inhibitors to undermine the signaling cascades for TNF-α release (Fig. 2).

Determination of Synergistic Impacts of TiO_2 Particles on Signaling Cascades for Proinflammatory Cytokine Release

A synergistic impact of TiO_2 particles in concert with a chemotherapeutic agent on the proinflammatory cytokine release from RAW 264.7 cells was investigated using our previously established "coculture system"[4] (Fig. 1).

The coculture system with RAW 264.7 and HeLa cells did not respond to the low dose of biotherapeutic agent itself, *i.e.*, 1 ng/ml PHA, 1 ng/ml LPS, 1 ng/ml ricin, or 1 ng/ml IFN-γ. In contrast, a considerable increase in the luminescence intensity in the coculture system was observed only when the cells were co-stimulated with 40-nm TiO_2 particles and 1 ng/ml IFN-γ. The co-stimulation induced 2.1-times-higher luminescence intensity from the cells than those in the absence of IFN-γ and TiO_2 particles (*i.e.*, $108 \pm 29\%$ increase). On the other hand, the low dose of PHA and LPS slightly varied the luminescence intensities in the presence of the 40-nm TiO_2 particles.

This synergistic effect of TiO_2 particles on proinflammatory cytokine release is interpreted that IFN-γ restores RAW 264.7 cells from a "rest" to an "activated" state through expressing adequate proteins for proinflammatory cytokine release. This view is supported by a previous study.[12] This result also suggests that the TiO_2 particles may affect, not only the signaling cascades for proinflammatory cytokine release, also other cascades of inflammatory responses activated with IFN-γ and LPS such as induction of cytostasis in living subjects.

ELISA-based Determination of the TNF-α Release

The total extent of TNF-α release was determined with an ELISA kit (Fig. 2). The stimulation with a vehicle (PBS, pH 7.2) as a control induced a basal secretion of *ca.* 297 pg/ml TNF-α from RAW 264.7 cells. This is a basal release of TNF-α in a physiological condition of the microphage. The basal secretion of TNF-α is caused by the pattern recognition receptors such as class A scavenger receptors on RAW 264.7 cells, which sense the hydrophobic, plastic bottom of the culture dish, as discussed previously.[13] The 40-nm TiO_2 particles (final conc: 50 μg/ml) increased the TNF-α release from RAW 264.7 cells up to *ca.* 505 pg/ml (*i.e.*, $70 \pm 4\%$ enhancement).

The signaling cascades by nanoscale TiO_2 particles upon secretion of TNF-α are categorized into three groups: *i.e.*, the signaling on the plasma membrane such as endocytosis (Group I), the signaling cascade beneath the plasma membrane, for instance, involving PI3-K (Group II), the further downstreams including MAPK pathway and NF-κB (Group III).

In Group I, monensin is known to collapse the pH gradient across endosomal membranes, which required trafficking of endocytosis vesicles and their subsequent acidification.[6] According to our results, monensin effectively inhibited the TNF-α release triggered by TiO_2 particles up to $55 \pm 3\%$. The additional comparison with the secretion level

of TNF-α in a TiO_2-free condition demonstrates the suppressive effect of TiO_2 particles on TNF-α release.

Dansylcadaverine is known to inhibit a transamidase required for clathrin-coated pit assembly at the initial step for endocytosis.[6] The ELISA results in Fig. 2 exhibit that dansylcadaverine greatly inhibited the TNF-α release from RAW 264.7 cells in both presence or absence of TiO_2 particles ($61 \pm 1\%$ and $49 \pm 3\%$, respectively). The magnitudes of the TNF-α release varied upon presence of TiO_2 particles.

These results demonstrate that TiO_2 particles trigger a series of endocytosis steps: clathrin-coated pit assembly, trafficking of endocytosis vesicle, and its subsequent acidification.

As a member of group II, a PKC inhibitor, H7,[7] was examined for determining the involvement of PKC in the TiO_2 particle-triggered TNF-α release. H7 weakly inhibited the basal TNF-α release (down to $16 \pm 0.4\%$). The inhibitory effect of H7 on the TNF-α release was interestingly enhanced in the presence of TiO_2 particles. The results show that activation of PKC pathway by TiO_2 particles is companied for TNF-α secretion.

The requirement of PI3-K for TNF-α release was estimated using wortmannin, known as a PI3-K inhibitor.[6] Wortmannin showed a weak inhibitory effect on the TNF-α release in the presence of TiO_2 particles ($37 \pm 2\%$ decrease). The result shows that TiO_2 particles modulate the TNF-α release via the PI3-K pathway.

We also explored the roles of tAG upon TNF-α release from RAW 264.7 cells in response to TiO_2 particles. tAG, which is known as an inhibitor for protein tyrosine phosphorylation, weakly blocked the TNF-α release triggered by TiO_2 particles ($23 \pm 4\%$ decrease). The result indicates that a protein tyrosine kinase weakly blocks TNF-α release in the presence of nanoscale TiO_2 particles.

The results with tAG and wortmannin demonstrate that the stimulation of TiO_2 particles to RAW 264.7 cells modulate the protein tyrosine phosphorylation and PI3-K activation. These results indicate the participation of some plasma membrane receptors that are connecting TiO_2 particles with the signaling cascades for TNF-α release. The receptors should include TLRs and/or growth factor receptors, which are known receptors for protein tyrosine phosphorylation and PI3-K activation.[14] Previously, TiO_2 particles were reported to activate NF-κB for TNF-α release via a pathway involving the EGF receptor.[15] Another study also represented that the downstream propagation of activated EGF receptor (EGFR) signaling involves at least two major pathways: the MAPK, and the PI3-K dependent pathway.[16] The previous studies on the signaling cascades were correspondently observed in our results in the present TiO_2 particle study.

A key intermediate in the MAPK pathway, MAPKK, is also an important intermediate in many receptor-mediated intracellular signaling pathways, the receptors of which include insulin receptor, epidermal growth factor receptor, TLR, and nerve growth factor receptor.[17] A well known MAPKK-specific inhibitor is U0126, which significantly blocked the TNF-α release in either presence or absence of TiO_2 particles ($78 \pm 3\%$ and $63 \pm 5\%$, respectively). The results strongly demonstrate that TNF-α production is directly connected to a MAPK pathway. In this case, the secretion levels of TNF-α were invariant in the presence or absence of TiO_2 particles.

In the overall results in Fig. 2, the most remarkable inhibitory effects appeared in the presence of dansylcadaverine (transamidase inhibitor), H7 (PKC inhibitor), and U0126 (MAPKK inhibitor). These selective adverse effects of the inhibitors on the TNF-α release strongly suggest that the TNF-α release is stimulated through endocytosis/PKC/MAPK pathways. Nevertheless, their unexpected blocking effects on other signaling cascades can not be completely excluded.

An inhibitory mechanism of glucocorticoids for TNF-α synthesis was reported previously in a glucocorticoid receptor (GR) study.[18] In practice, corticosterone as an endogenous glucocorticoid exhibited a strong inhibitory effect on the secretion of TNF-α in the presence or absence of TiO_2 particles ($65 \pm 2\%$ and $71 \pm 3\%$, respectively). The effect is interpreted as the corticosterone-activated GR inhibits the basal TNF-α secretion caused by pattern recognition receptors on RAW 264.7 cells, which sense the hydrophobic plastic culture dish.

Previously, the contribution of an individual biotherapeutic agent to inflammation was estimated from the expressed levels of cytokines from macropharge cells using ELISAs.[19] Synergistic effects of multiple biotherapeutic agents on inflammation were also explored with the same methods.[5] However, the combined effects of nanoparticles and biotehrapeutic agents were not estimated on the signaling cascades of inflammation.

Western Blot for Determining TNF-α Release

The influence of TiO_2 particles on the TNF-α release in the presence of IFN-γ was explored using Western blot analysis (Fig. 2, Inset). The TNF-α release into the culture medium was greatly increased by raising the concentration of TiO_2 particles in the presence of IFN-γ. On the other hand, the total TNF-α levels inside the RAW 264.7 cells were invariant according to the concentrations of TiO_2 particles. The result demonstrates that RAW 264.7 cells upon sensing TiO_2 particles immediately secrete TNF-α. The blot in Line 4 appeared in the thickest band. The blots demonstrate not the absolute, but the relative amounts of the TNF-α release from RAW 264.7 cells.

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