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Effects of Toll-like Receptors 3 and 4 Induced by Titanium Dioxide Nanoparticles in DNA Damage-Detecting Sensor Cells

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Abstract

Live cell-based sensor reporter systems (so-called sensor cells) were employed to detect host defense systems, including DNA damage response, stimulated by nanoparticles (NPs). Our previous work established the use of DNA damage-detecting sensor cells containing the B-cell translocation gene 2(BTG2) promoter-reporter plasmid and showed that Toll-like receptors (TLRs) are involved in the cellular response and uptake of TiO_2 NPs. These results suggested that TLRs could be involved in many cellular responses. However, the effect of TLRs on DNA damage induced by TiO_2 NPs is unknown. Here we investigated the role of TLR 3 and 4 in DNA damage induced by PEG-modified TiO_2 and by TiO_2 NPs. The results show that PEG modification of TiO_2 NPs reduces DNA damage response compared to unmodified TiO_2 NPs. The overexpression of TLR3 reduces DNA damage mediated by both TiO_2 and PEG-TiO_2 NPs. In contrast, overexpression of TLR4 increases the DNA damage response induced by TiO_2 NPs. The results indicate that co-transfection of TRL4 expression vector affects the sensitivity of DNA damage response, but does not affect the detection limit of the DNA damage response. These finding will aid in understanding the molecular interaction mechanisms between NPs and cells.

Keywords: Titanium dioxide nanoparticles; PEG-TiO_2 modification; Live cell-based biosensors; DNA damage response; Toll-like receptors

Introduction

Nanoparticles (NPs) are tiny particles (diameter of 1 to 100 nm in at least one dimension) [1] characterized by a very high surface area-to-volume ratio [2]. Due to the unique properties afforded by their size, NPs possess a wide range of applications in the industrial, electrical, agricultural, pharmaceutical, and medical fields. Titanium dioxide (TiO_2) NPs are used as a photocatalyst [3] for cleaning air and water, and are found in a wide array of products including paints, pigments, cosmetics, and skin care products [4]. TiO_2 NPs are classified as a biologically inert substance in animals and humans [5,6]. Recent findings have revealed that rats exposed to ultra-fine TiO_2 NPs develop inflammation, pulmonary damage, and lung tumors [7,8]. This toxicity may be due to the ease with which these NPs can pass through the cell membrane and disrupt biological systems [9]. It has been suggested that the small size and corresponding high specific surface area are the major determinants of NP toxicity [10]. It has also been proposed that the high surface area of NPs greatly increases their ability to produce potentially toxic Reactive Oxygen Species (ROS) [11].

Live cell-based sensor reporter systems (so-called sensor cells) have been employed to study host defense systems, including innate immune response, environmental stress response and DNA damage response, stimulated by NPs. The attraction of systems employing sensor cells is that they are highly sensitive and effective compared with traditional methods [12]. Our previous work established three kinds of live cell-based sensor reporter systems: a nuclear factor kappa B (NF-κB) reporter system [13], a Heat Shock Protein (HSP) reporter system [14,15] and a B-cell translocation gene 2 (BTG2) system [16]. These results suggested that these three sensor cells hold promise for detecting cellular response to NPs.

Polyethylene Glycol (PEG) is a coiled polymer of repeating ethylene ether units with a dynamic conformation. PEG is inexpensive, versatile and FDA-approved for many applications [17]. In addition, PEG is non-toxic and non-immunogenic, and has favorable pharmacokinetics and tissue distribution [18]. Modifying the surface of NPs with PEG (PEGylation) not only prevents agglomeration [19], but also renders NPs resistant to protein adsorption and enhances their biocompatibility [20]. Coating nanomaterials with PEG also increases the *in vivo* circulation time, thereby likely reducing clearance via the ReticuloEndothelial System (RES) [21]. We have already shown that PEGylation of TiO_2 NPs reduces cytotoxity and inflammatory response [22]. The results suggested that PEGylation of TiO_2 NPs could reduce many cellular responses. However, the effect of PEGylation of TiO_2 NPs on cellular DNA damage is unknown.

DNA damages are abnormal chemical and structural alterations, mutations ordinarily involve the normal four bases in new arrangements. Cellular DNA damage is caused by chemicals or ionizing radiation and can lead to proliferation and cancer. The most important guardian of the genome is p53, a tumor suppressor protein. p53 triggers cellular outcomes through its role as a sequence-specific DNA-binding transcriptional factor of genes involved in regulation of the cell cycle, apoptosis, and DNA repair in response to DNA damage [23,24]. B-cell translocation gene 2 (BTG2) is involved in cell-growth control, and BTG2 expression is regulated by p53 [25]. BTG2 expression is

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upregulated by p53 after DNA damage induced by a genotoxic agent [26]. We have established sensor cells for DNA damage detection using BGT2 promoter-reporter plasmid (DNA damage-detecting sensor cells) [27], and have shown that this type of sensor cell can detect DNA damage induced by TiO_2 NPs [16].

Toll-like receptors (TLRs) play an essential role in the activation of innate immunity by recognizing specific molecular patterns of microbial components. TLRs are transmembrane proteins that comprise both an extra-cellular domain (responsible for ligand recognition) and a cytoplasmic domain (required for initiating signaling) [28]. As suggested by their range of ligands and subcellular locations, TLRs recognize a wide range of 'foreign' materials [29,30]. We have previously shown that TLRs are also involved in the cellular response and cellular uptake of TiO_2 NPs [31,32]. These results suggested that TLRs could be involved in many cellular responses. However, the effect of TLRs on DNA damage induced by TiO_2 NPs is unknown.

In this investigation we studied the role of TLR3 and TLR4 in cellular DNA damage response induced by exposure to TiO_2 and PEG-modified TiO_2 NPs (PEG-TiO_2 NPs). The aim of this work was to improve the sensitivity and detection limit of DNA damage-detecting sensor cells following transfection with TLR3 and TLR4 expression vectors. The results indicate that co-transfection with TRL4 expression vector affects the sensitivity of DNA damage-detecting sensor cells, but does not affect the detection limit of the DNA damage response. This information is important for the detection of nano-toxicological response.

Materials and Methods

Cells and cell culture

The human hepatocellular carcinoma cell line, HepG2, was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaillé, France, UK), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque, Inc.,) at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmids employed

pGL3-Control vector (pGL3 plasmid; Promega, Madison, WI, USA) was employed as an 'empty' control reporter plasmid. BTG2 promoter-reporter plasmid (the region from nt -100 to -20 bp of the BTG2 gene containing the p53 binding site mutation [27]), and TLR3 and TLR4 promoter-reporter plasmids, were used to detect the DNA damage response. All the reporter plasmids contain SV40 promoters and enhancer sequences, resulting in strong expression of the luciferase-encoding gene (luc^+) in many types of mammalian cells. The pRL-CMV vector (CMV, Renilla luciferase-encoding control plasmid; Promega) contains the CMV promoter upstream of the Renilla luciferase gene and was used as an internal control for variations in transfection efficiency. TLR-encoding genes were purchased from InvivoGen (San Diego, CA, USA). The pUNO1-mcs expression vector was used as an 'empty' control vector. Since pUNO1-mcs does not contain a therapeutic gene, it can be used in conjunction with other vectors of the pUNO1 family to serve as an experimental control. Overproduction of TLR3 and TLR4 was provided by transfection with pUNO-hTLR3 (which encodes the human TLR3 protein), and pUNO1-hTLR04a (CD284a) (which harbors the human TLR04a (CD284a) encoding open reading frame), respectively.

Construction of DNA damage-detecting sensor cells

Reporter plasmid (blank control reporter, pGL3 plasmid or BTG2 promoter-reporter plasmid) and internal control plasmid (pRL-CMV vector) were co-transfected into HepG2 cells. The transfection was performed with Lipofectamine™ LTX Reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. HepG2 cells were seeded in 24-well plates. After overnight incubation, the cells were co-transfected with the plasmids using Lipofectamine™ LTX Reagent. For co-transfection experiments, reporter plasmid (blank control reporter, pGL3 plasmid or BTG2 promoter-reporter plasmid), TLR3 or TLR4 expression vectors and internal control plasmid (pRL-CMV vector) were co-transfected into HepG2 cells.

Preparation of and exposure to TiO_2 NP

The preparation and characterization of TiO_2 NPs were described in previous studies [13,14]. Briefly, nano-TiO_2 (AeroxideR P25; Sigma-Aldrich, St Louis, MO, USA) was dispersed in distilled water and autoclaved at 120°C for 20 min. The suspension was cooled to room temperature and then sonicated for 10 min at 200 kHz using a high-frequency ultrasonic sonicator (MidSonic 600, Kaijo Corp., Tokyo, Japan). The resulting nano-TiO_2 suspension was designated 'TiO_2 NPs'. The concentration of TiO_2 NPs was determined using a UV–vis spectrophotometer (UV-1600, Shimadzu, Kyoto, Japan). The suspension was adjusted to the desired concentration by the addition of distilled water and stored at 4°C until use. The particle size distribution was measured by dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Malvern, UK). The aggregated particle size of the TiO_2 NPs was determined to be 216 ± 70 nm. The size of the aggregated TiO_2 NPs remained stable for several weeks under the indicated storage conditions. Prior to addition to the cell cultures, the suspension of TiO_2 NPs was diluted into supplemented medium and used as described above. For the reporter gene (transfected cell) assays, the culture medium was replaced (1 day after transfection) with medium containing the TiO_2 NPs at the intended concentration. Specifically, TiO_2 NPs were added to the culture medium immediately before the medium was applied to the cells. After the indicated exposure times, the cells were harvested and assayed for luciferase activity. Polyethylene glycol nanoparticles (PEG-TiO_2) were prepared as described previously [33].

Luciferase activity assessment

The luciferase activities were assessed by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA), as described previously [13,14]. Following TiO_2 NP exposure, the cells were lysed in 1×passive lysis buffer, then luciferase and *Renilla* light units were measured using a Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) luminometer according to the manufacturer's protocol for the Dual Luciferase assay. All the results represent at least three independent tests. Data are expressed as means ± Standard Deviations (S.D.).

Results and Discussion

We have previously shown that uncoated TiO_2 NP aggregates induce DNA damage response [16]. Botelho et al. also have shown that TiO_2 NPs induce DNA damage response and participate in a number of carcinogenesis-mediated processes, such as increased cell proliferation, decreased apoptosis and increased oxidative stress in human gastric epithelial cells *in vitro* [34]. In order to reduce the DNA damage response, we conjugated TiO_2 NPs with polyethylene glycol (PEG). In this study, a live cell-based biosensor based on a B-cell Translocation

Gene 2 (BTG2) promoter-reporter was used to detect DNA damage response induced by PEG-TiO_2 NPs and TiO_2 NPs in BTG2 promoter-reporter plasmid transfected HepG2 cells. In previous work, a BTG2 biosensor for the BTG2 promoter response detected the cytotoxicity caused by DNA strand breaks with high sensitivity [27]. As shown in Figure 1, TiO_2 NPs and PEG-TiO_2 NPs induced DNA damage response. HepG2 cells exposed to PEG-TiO_2 NPs showed a 2.2 times higher DNA damage response compared to the control, while cells exposed to TiO_2 NPs showed a 4.9 times higher DNA damage response compared with the control. The results indicate that PEG modification reduces DNA damage response induced by TiO_2 NPs.

Toll-like receptors (TLRs) recognize and respond to exogenous and endogenous ligands through signaling pathways, leading to inflammatory cascade mediator production which directs the innate and adaptive immune response. TLRs are conserved membrane-bound Pattern Recognition Receptors (PRRs) that recognize a broad spectrum of microbial components such as lipopeptides and non-self nucleic acids [35]. We have previously shown that TLRs are involved in TiO_2 NP cellular uptake [31,32]. In order to investigate the effect of TLRs on DNA damage response induced by NPs, live cell-based sensor cells incorporating the BTG2 promoter-reporter, and either the TLR 3 or TLR4 expression vector, were used to detect the DNA damage response induced by PEG-TiO_2 NPs and TiO_2 NPs. The concentration of both NPs was standardized at 10 μg/ml and the exposure times ranged from 3 h to 48 h. As shown in Figure 2, HepG2 cells transfected with TLR3 and exposed to TiO_2 NPs showed decreased BTG2 response compared to cells without TLR transfection. In contrast, HepG2 cells transfected with TLR4 showed a higher BTG2 response, as measured by the BTG2 promoter-luciferase reporter plasmid, compared to HepG2 cells without TLR transfection. The highest BTG2 response of cells transfected with TLR4 compared to cells without TLR transfection was at 48 h exposure to the TiO_2 NPs. The response of cells transfected with TLR4 was 5.5 times that of cells transfected with TLR3, and that of the control cells was 3.9 times that of cells transfected with TLR3 (Figure 2). The results indicate that TLR4 enhanced DNA damage induced by TiO_2 NPs and that TLR3 reduced DNA damage induced by TiO_2 NPs.

On the other hand, HepG2 cells transfected with TLR4 and exposed to PEG-TiO_2 NPs showed no effect (Figure 3), while cells transfected with TLR3 showed a decrease in BTG2 response compared with non-transfected cells. The highest BTG2 response (2.1 times compared to the control) occurred after 48 h exposure to PEG-TiO_2 NPs. The results indicate that TLR4 did not enhance DNA damage induced by PEG-TiO_2 NPs, and that TLR3 reduced DNA damage induced by PEG-TiO_2 NPs.

In order to compare the effect of TLR4 on the sensitivity and detection limit of DNA damage response, the dependence of the DNA damage response on TiO_2 NP concentration was investigated. It is clear from Figure 4a that an increase in the concentration of TiO_2 NPs increased the BTG2 response of HepG2 cells transfected with BTG2 promoter-luciferase reporter plasmid with or without transfection with the TLR4 expression vector. The highest BTG2 response was at 10 μg/

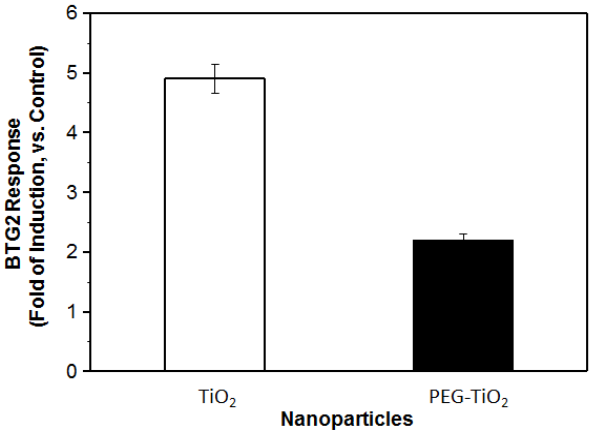

Figure 1: Comparison of BTG2 response induced by TiO_2 and PEG–TiO_2 NPs compared to the control (cells not exposed to NPs). Cells were exposed to 10 μg/mL of either TiO_2 or PEG–TiO_2 NPs for 48 h. Each column was produced from at least 3 replicate measurements. All values are presented as mean ± S.D. (n ≥ 3).

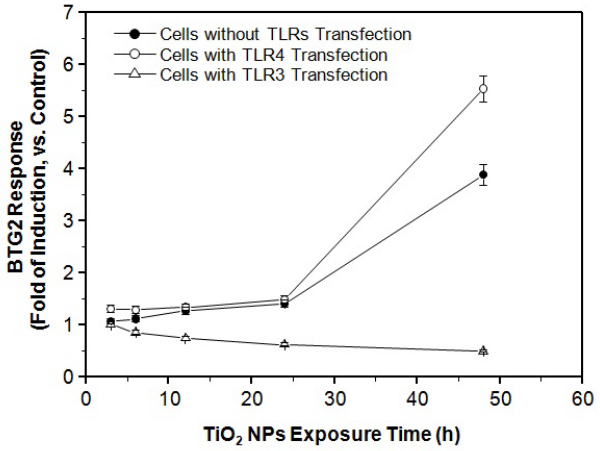

Figure 2: Time course of HepG2 exposure to TiO_2 NPs. The cells were transfected with BTG2 promoter-reporter plasmid and co-transfected with or without TLR3 and TLR4 expression vectors. The transfected cells were exposed to 10 μg/mL TiO_2 NPs for the indicated lengths of time. Each plot was produced from at least 3 replicate measurements. All values are presented as mean ± S.D. (n ≥ 3).

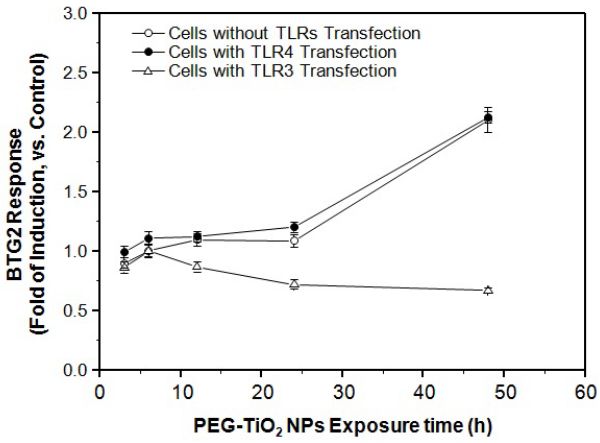

Figure 3: Time course of HepG2 exposure to PEG–TiO_2 NPs. The cells were transfected with BTG2 promoter-reporter plasmid and co-transfected with or without TLR3 and TLR4 expression vectors. The transfected cells were exposed to 10 μg/mL TiO_2 NPs for the indicated lengths of time. Each plot was produced from at least 3 replicate measurements. All values are presented as mean ± S.D. (n ≥ 3).

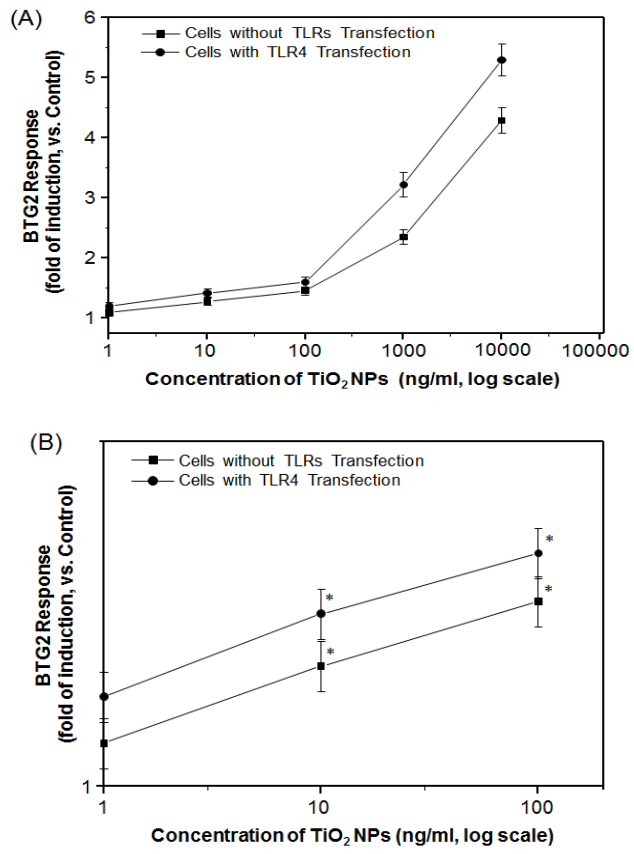

Figure 4: (a) Effect of TLR4 on BTG2 response (fold of induction) of HepG2 cells transfected with BTG2 promoter-luciferase reporter plasmid and TLR4 expression vector. (b) An expanded view of the response at low concentrations of TiO_2 NPs. Transfected cells were exposed to the indicated concentrations of TiO_2 NPs for 48 h. The results are shown as mean ± S.D., n ≥ 3 for each concentration.

ml, and cells transfected with TLR4 showed a higher BTG2 response (5.2 times higher compared to cells not transfected with TLRs). This result indicated that co-transfection with TLR4 expression vector increases sensitivity towards DNA damage response. Figure 4b shows an expanded view of the BTG response of HepG2 cells exposed to low concentrations TiO_2 NPs. The detection limit of DNA damage response by HepG2 cells transfected with BTG2 promoter-reporter plasmid with or without TLR4 expression vector was 10 ng/ml of TiO_2 NPs. The results indicate that co-transfection with TRL4 expression vector does not affect the detection limit of the DNA damage response.

In the present study, we focused on the roles of TLR3 and TLR4 in the DNA damage response induced by PEG-TiO_2 and TiO_2 NPs. TLRs have been studied for their role in the recognition of microbial pathogens. Each TLR recognizes a specific pathogen-associated pattern. For example, TLR 3 localizes to the endosome surface and recognizes viral double-stranded RNA [36], whereas TLR 4 localizes to the cell surface and binds with Gram-negative bacterial cell wall components such as LPS [37]. Transfection of HepG2 cells with TLRs could change the DNA damage response induced by TiO_2 and PEG-TiO_2 NPs. We hypothesize that transfection with TLR4 expression vector elevates the level of TLR4 on the cell surface, thus increasing the uptake of TiO_2 NPs in the cytoplasm and hence increasing the risk of DNA damage response. On the other hand, elevated levels of subcellular TLR3 could combine with unengaged TiO_2 and PEG-TiO_2 NPs in the cytoplasm and retain those NPs in the endosome, thereby reducing the risk of cytotoxicity and DNA damage mediated by both TiO_2 and PEG-TiO_2 NPs. Hence, the cellular locations of TLR3 and TLR4 could result in opposing effects on DNA damage response induced by TiO_2 NPs.

Conclusions

Our results show that PEG modification of TiO_2 causes reduced DNA damage response compared with TiO_2 NPs. The overexpression of TLR3 reduced DNA damage mediated by both TiO_2 and PEG-TiO_2 NPs. In contrast, overexpression of TLR4 increased the DNA damage response induced by TiO_2 NPs. Our results indicate that co-transfection of TRL4 expression vector affects the sensitivity of the DNA damage response, but does not affect the detection limit of the DNA damage response.

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