Titanium Dioxide Nanoparticles Induce DNA Damage and Genetic Instability In vivo in Mice

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Introduction

Titanium dioxide (TiO2) nanoparticles are manufactured worldwide in large quantities for use in a wide range of applications including pigment and cosmetic manufacturing. Although TiO2 is chemically inert, TiO2 nanoparticles can cause negative health effects, such as respiratory tract cancer in rats. However, the mechanisms involved in TiO2-induced genotoxicity and carcinogenicity have not been clearly defined and are poorly studied in vivo. The present study investigates TiO2 nanoparticles–induced genotoxicity, oxidative DNA damage, and inflammation in a model. We treated wild-type mice with TiO2 nanoparticles in drinking water and determined the extent of DNA damage using the comet assay, the micronuclei assay, and the γH2AX immunostaining assay and by measuring 8-hydroxy-2′-deoxyguanosine levels and, as a genetic instability endpoint, DNA deletions. We also determined mRNA levels of inflammatory cytokines in the peripheral blood. Our results show that TiO2 nanoparticles induced 8-hydroxy-2′-deoxyguanosine, γH2AX foci, micronuclei, and DNA deletions. The formation of γH2AX foci, indicative of DNA double-strand breaks, was the most sensitive parameter. Inflammation was also present as characterized by a moderate inflammatory response. Together, these results describe the first comprehensive study of TiO2 nanoparticles–induced genotoxicity in vivo in mice possibly caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress. Given the growing use of TiO2 nanoparticles, these findings raise concern about potential health hazards associated with TiO2 nanoparticles exposure. [Cancer Res 2009;69(22):8784–9]

Abstract

Titanium dioxide (TiO2) nanoparticles are manufactured worldwide in large quantities for use in a wide range of applications including pigment and cosmetic manufacturing. Although TiO2 is chemically inert, TiO2 nanoparticles can cause negative health effects, such as respiratory tract cancer in rats. However, the mechanisms involved in TiO2-induced genotoxicity and carcinogenicity have not been clearly defined and are poorly studied in vivo. The present study investigates TiO2 nanoparticles–induced genotoxicity, oxidative DNA damage, and inflammation in a model. We treated wild-type mice with TiO2 nanoparticles in drinking water and determined the extent of DNA damage using the comet assay, the micronuclei assay, and the γH2AX immunostaining assay and by measuring 8-hydroxy-2′-deoxyguanosine levels and, as a genetic instability endpoint, DNA deletions. We also determined mRNA levels of inflammatory cytokines in the peripheral blood. Our results show that TiO2 nanoparticles induced 8-hydroxy-2′-deoxyguanosine, γH2AX foci, micronuclei, and DNA deletions. The formation of γH2AX foci, indicative of DNA double-strand breaks, was the most sensitive parameter. Inflammation was also present as characterized by a moderate inflammatory response. Together, these results describe the first comprehensive study of TiO2 nanoparticles–induced genotoxicity in vivo in mice possibly caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress. Given the growing use of TiO2 nanoparticles, these findings raise concern about potential health hazards associated with TiO2 nanoparticles exposure. [Cancer Res 2009;69(22):8784–9]
Thus far, most nanoparticle genotoxicity studies have focused on cell culture systems, but confirmation from animal experiments, more relevant to human exposure, is required. To further understand TiO₂ nanoparticles toxicity in vivo, we studied the effect of TiO₂ nanoparticles exposure on genotoxicity, DNA damage, and inflammation in mice. To evaluate inflammation in mice, we determined mRNA expression of both proinflammatory and anti-inflammatory cytokines. To assess DNA damage, we used the γH2AX and the comet assays to evaluate DNA strand breaks, the micronucleus assay to estimate chromosomal damage, and the 8-OHdG, γH2AX foci, micronuclei, DNA deletions, and inflammation markers in a mice model. Therefore, this study suggests that TiO₂ nanoparticles are genotoxic in vivo.

### Materials and Methods

#### Mouse care and breeding.
C57Bl/6j/p<sup>pm</sup>/p<sup>pm</sup> mice were obtained from The Jackson Laboratory. The C57Bl/6j/p<sup>pm</sup>/p<sup>pm</sup> background is essentially identical to C57Bl/6j, with the exception of a naturally occurring 70-kb internal duplication in the pink-eyed dilution (p) gene, termed the p<sup>pm</sup> allele. Mice were housed and cared for under standard specific pathogen-free conditions and according to the Animal Rescue Coalition and Institutional Animal Care and Use Committee regulations. Mice were given a standard, ad libitum drinking water. Pregnant mice were timed by checking for vaginal plugs, with no of the day of discovery counted as 0.5 days post-coitum. Four- to 5-month-old mice were used for all experiments.

#### TiO₂ nanoparticles preparation and exposure.
"Aeroxide" P25 TiO₂ (Degussa, now Evonik) nanoparticles were chosen for this study. The crystal structure is a mixture of 75% anatase and 25% rutile TiO₂, purity was at least 99.5% TiO₂, and primary particle size was 21 nm with a specific surface area of 50 ± 15 m²/g. These nanoparticles have been used in many of the previous mammalian studies (13, 14, 22, 25–28). Using dynamic light scattering in water revealed that the size of TiO₂ nanoparticles agglomerates ranged from 21 to 1,446 nm and the mean size was 160 ± 5 nm. About 70% of particles have a size of 160 nm. Solutions of dispersed TiO₂ nanoparticles were prepared by ultrasonication (Solid State/Ultrasonic FS-14; Fisher Scientific) for 15 min in drinking water at 60, 120, 300, and 600 μg/mL concentrations just before use. We measured TiO₂ nanoparticles–supplemented water intake at the end of experiments in each cage, which housed 2 to 3 mice, and calculated an average daily water intake per mouse. Daily TiO₂ nanoparticles–supplemented water intake ranged from 3 to 7 mL/mouse, consistent with normal daily water intake. Doses were calculated using a 30 g average weight per mouse, and an average of 5 mL water intake per day. The exposure was 5 days in adult males. For in utero exposure, pregnant dams were given nanoparticles–supplemented drinking water for 10 days from 8.5 to 18.5 days post-coitum at a concentration of 300 μg/mL. Water was used as negative control.

#### In vivo DNA deletion assay.
To evaluate genotoxicity of TiO₂ nanoparticles, we employed an intrachromosomal duplication of 70-kb fragment spanning exons 6 to 18 of the p gene in mice (termed p<sup>pm</sup> mutation). When a DNA deletion event occurs between these duplications, the p<sup>pm</sup> allele reverts to the wild-type p gene. Reconstitution of the wild-type p gene can be seen as a single pigmented cell or a clone of pigmented cells on the unpigmented retinal pigment epithelium (RPE) in the transgenic mice and represents a DNA deletion as a permanent genotoxic event (29). Pregnant mice were treated with TiO₂ nanoparticles, and offspring were sacrificed at age 20 days. Their eyes were extracted and dissected to display the RPE for the deletion/eyespot assay as described previously (24). One RPE corresponds to one eye.

#### Peripheral blood.
Peripheral blood was collected by submandibular vein puncture (before treatment and after treatment) in an EDTA-coated tube. The comet assay was done as described previously (30). On average, from three slides, 150 to 200 randomly captured comets per sample were analyzed. Results were expressed as average ± SE tail moment.

#### Micronucleus assay.
The micronucleus assay was done as described elsewhere (31). Three microaliquots of the peripheral blood were collected as described above and smeared on slides and stained into Giemsa stain for 1.5 min. Approximately 2,000 erythrocytes were scored per animal to estimate the frequency of micronucleated erythrocytes.

#### Bone marrow preparation.
Animals were sacrificed with an overdose of isoflurane after 5 days of treatment with TiO₂ nanoparticles in drinking water. Both femora were dissected, and marrow cells were flushed out with 1 mL PBS and pipetted several times. The cell suspension was centrifuged at 1,000 rpm for 5 min. The supernatant was withdrawn, and the cell pellet was resuspended and placed on a clean glass slide.

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**Figure 1.** Frequency of DNA deletions in control and TiO₂ nanoparticles–treated mice. One RPE corresponds to one eye. Mice were treated with nanoparticles during embryonic development at a total dose of 500 mg/kg. Mean ± SE numbers of eyespots per RPE with n = 42 eyes for control and n = 53 eyes for TiO₂ nanoparticles–treated mice. *, P < 0.05.

**Figure 2.** Percentage of γH2AX–positive cells in bone marrow in untreated and TiO₂ nanoparticles–treated mice and a picture of a γH2AX–positive cell with more than four foci. Columns, mean of 5 mice; bars, SE. ***, P < 0.001. TiO₂ nanoparticles–treated versus control.
Frequency of DNA strand breaks in mice before and after treatment with 500 mg/kg TiO₂ nanoparticles. DNA damage is represented by the tail moment. Mean ± SE (n = 5 mice/group). *, P < 0.05, compared with untreated mice.

**Results**

**TiO₂ nanoparticles increased the frequency of DNA deletions.** We used the DNA deletion assay to evaluate in vivo genotoxicity of TiO₂ nanoparticles. We quantified the number of eye spots per RPE as a measure of DNA deletions in in utero exposed mice. TiO₂ nanoparticles–treated mice had an average of 8.13 ± 1.70 eye spots per RPE versus 6.42 ± 1.47 eye spots per RPE in nontreated mice (Fig. 1). TiO₂ nanoparticles–exposed mice displayed a significant increase in eye spots (27%) compared with untreated mice (P = 0.019), suggesting that, after maternal oral exposure, TiO₂ nanoparticles increased DNA deletion frequency in fetuses.

**TiO₂ nanoparticles induced γ-H2AX foci.** Phosphorylation of histone H2AX on serine 139 occurs at sites flanking DNA double-strand breaks (DSB), providing a measure of the number of DSBs within a cell (33). We used this assay to compare DSB formation in bone marrow of mice with and without TiO₂ nanoparticles treatment.

The γ-H2AX foci formation increased by ∼10%, 20%, 25%, and 30% following treatment with 50, 100, 250, and 500 mg/kg TiO₂ nanoparticles, respectively, compared with untreated mice (Fig. 2). Percentage of γ-H2AX–positive cells increased with TiO₂ nanoparticles concentration in a clear dose-dependent manner (P < 0.001). These data provided evidence that, after oral administration, TiO₂ nanoparticles induce DSBs in bone marrow cells.

**TiO₂ nanoparticles increased DNA strand breaks.** DNA stand breaks (DSBs, single-strand breaks, and/or strand breaks induced by alkali-labile sites) were measured by the alkaline comet assay in mice peripheral blood before and after treatment. Tail moment significantly increased after TiO₂ nanoparticles treatment (Fig. 3). The average tail moment was 0.0102 ± 0.001 before treatment and 0.0137 ± 0.0011 after TiO₂ nanoparticles treatment. TiO₂ nanoparticles increased DNA strand breaks in WBCs from peripheral blood by 34% (P = 0.001, t test, and P = 0.04, Wilcoxon test).

**Statistical analysis.** For the deletion assay, the comet assay, the micronuclei assay, and 8-OHdG and mRNA levels of cytokines, we used the Student’s t test to compare untreated mice with treated mice. For the γ-H2AX experiment, the percentage of positive cells for control groups versus treated groups was compared via the χ² test. In addition to the t test, for comet assay data, the Wilcoxon test for matched paired data was also used to compare the effect of TiO₂ nanoparticles on the tail moment before and after nanoparticle treatment. The difference was considered significant at the 95% confidence level (P < 0.05) and highly significant at the 99% confidence level (P ≤ 0.01).

**TiO₂ nanoparticles induced oxidative DNA damage.** We examined the degree of oxidative DNA damage by measuring the level of 8-OHdG in DNA isolated from TiO₂ nanoparticles–treated and untreated mouse livers. The level of 8-OHdG was significantly higher in TiO₂ nanoparticles–treated mice than untreated mice (P = 0.04; Fig. 5). The average number of 8-OHdG per 10⁶ dG was 4.25 ± 0.66 for untreated mice and 6.43 ± 0.58 for TiO₂ nanoparticles–treated mice resulting in a 1.5-fold increase at 500 mg/kg TiO₂ nanoparticles. This suggested that TiO₂ nanoparticles induced oxidative DNA damage in liver.

**Results**

**TiO₂ nanoparticles induced DNA damage.** We quantified mRNA transcripts of three Th1/proinflammatory cytokines (T-helper cell type 1) and three Th2/anti-inflammatory cytokines (T-helper cell type 2) in the peripheral blood. After treatment, the proinflammatory cytokines tumor necrosis factor-α, IFN-γ, and the mouse orthologue of interleukin-8 (KC) were significantly upregulated (P = 0.01, 0.02, and 0.05, respectively; Fig. 6A).

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A general upregulation of these cytokines may be due to the effects of circulating TiO2 nanoparticles directly in the peripheral blood, suggesting systemic distribution, and direct activation of a proinflammatory response. In the contrary, anti-inflammatory cytokines with generally opposing function were not upregulated, including transforming growth factor-\(\beta\), interleukin-10, and interleukin-4 (Fig. 6B). TiO2 nanoparticles did not induce an anti-inflammatory response, which mean they did not inhibit the production and release of proinflammatory mediators.

Discussion

Here, we report for the first time that TiO2 nanoparticles are genotoxic and clastogenic in vivo in mice. We showed that TiO2 nanoparticles (500 mg/kg) induce not only DNA single-strand breaks and DSBs but also chromosomal damage. The formation of \(\gamma\)-H2AX foci, which show DSB formation, was the most sensitive parameter and showed a consistent dose-dependent response. Concerning health relevance, DSBs are much more damaging in terms of genetic instability than single-strand breaks and oxidative DNA damage, which are transient. Our results extend previous in vitro findings with the micronuclei and comet assays in several human cells and Syrian hamster embryo cells (10, 12, 13, 15), although they have not been detected in some studies (11, 14). Differences in response between studies may be due to how TiO2 nanoparticles differ in terms of TiO2 production, particle size, degree of aggregation, preparation method (sonication), incubation conditions, dose, and susceptibility between cell types (34, 35), implying that more studies are needed to determine the conditions in which TiO2 nanoparticles genotoxicity occurs.

To date, very few in vivo genotoxicity studies have been carried out with nanoparticles. A chronic exposure to TiO2 nanoparticles at concentrations that produce chronic pulmonary inflammation was associated with an increased incidence of tumors in rat lungs (3). Thus far, only two in vivo genotoxicity studies have been reported, which showed that in vivo TiO2 nanoparticles increased Hprt mutation frequency in alveolar epithelial cells (9) but did not induce DNA adduct formation in rat lungs (26). Our study showed for the first time that, in vivo after oral exposure, TiO2 nanoparticles induce DNA strand breaks and chromosomal damage in bone marrow and/or peripheral blood, which may help to further understand potential mechanisms of TiO2 nanoparticles carcinogenicity. We also found that maternal exposure to 500 mg/kg TiO2 nanoparticles during gestation results in significantly elevated frequencies of DNA deletions in offspring. This result is a major finding because it shows for the first time that in utero exposure of fetuses via the mother causes an increase in large deletions in offspring. Taken together, our findings show that TiO2 nanoparticles, orally administrated, induce genotoxicity systemically in organs, such as blood, bone marrow, and even the embryo.

Surprisingly, human studies have not been able to detect any relation between TiO2 occupational exposure and cancer risk (4, 5, 36), but these studies have methodologic and epidemiologic limitations as reviewed by the National Institute of Occupational Safety and Health (37). In addition, these studies were not designed to investigate the relationship between TiO2 particle size and lung cancer risk, an important question for assessing the potential occupational carcinogenicity of TiO2. Indeed, Dankovic and colleagues, comparing several tumor studies, pointed out that TiO2 nanoparticles produced lung tumors in rats at a much lower dose than fine particles (<250 nm; 10 and 250 mg/m\(^3\) for nanoparticles and fine particles, respectively; ref. 3). Although TiO2 nanoparticles are prone to forming agglomerates of >100 nm in solution, these agglomerates are apparently not stable and appear to dissociate in

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Level of 8-OHdG in untreated and 500 mg/kg TiO2 nanoparticles–treated mouse livers. Mean ± SE (\(n = 5\) mice/group). *, \(P < 0.05\), compared with untreated mice.

![Figure 6](https://example.com/figure6.png)

**Figure 6.** TiO2 nanoparticles at 500 mg/kg induce proinflammatory but not anti-inflammatory cytokines. Open columns, untreated controls; black columns, TiO2 nanoparticles–treated mice. A, expression of proinflammatory cytokine panel relative to TBP, the internal control gene. *, \(P < 0.05\); **, \(P < 0.01\), Student’s \(t\) test for treatment comparisons. B, expression of anti-inflammatory cytokine panel relative to TBP. Student’s \(t\) test revealed no significant differences. For each graph, \(n = 5\) mice/group.
bodily fluids and tissues, which could be an explanation for TiO2 nanoparticles higher toxicity. It has also been reported that TiO2 nanoparticles surface interactions are weak (20). In addition, an inhaled study showed that TiO2 nanoparticles agglomerates of ~700 nm dissociate into smaller units after deposition in the lung (21). Even if nanoparticles aggregate into larger-sized agglomerates, their primary particle sizes remain a significant trait that affects their toxicity. Further human studies would be necessary to understand nanoparticle health effects. For instance, one could use blood-based assays similar to those done in this study in a future molecular epidemiology study in occupational settings. Furthermore, TiO2 is also used in food colorants and toothpaste. This supports the notion that nanoparticle ingestion constitutes a relevant route of exposure in humans and underscores the significance of the findings of our study. In addition, given the capability of nanoparticles to enter the systemic blood circulation, nanoparticles may pose hazard to a variety of other organs as we have shown here.

As suggested previously, a possible mechanism for nanoparticles caused genotoxicity might be via oxidative stress (38). Indeed, previous studies showed that TiO2 nanoparticles have hydroxyl radical activity (39–41) and can also trigger reactive oxygen species production in different cell lines (13, 42) on interaction with the cell membrane or even in cell-free environment (23, 43). We confirmed these results in our in vivo experiment by showing oxidative DNA damage (8-OHdG) increase in mouse livers after TiO2 nanoparticles treatment. Also, TiO2 nanoparticles–increased DNA deletions during fetal development might be a result of oxidative genome damage. As discussed previously (44), oxidative stress is particularly hazardous in replicating cells. For instance, oxidative DNA lesions (e.g., 8-OHdG, single-strand breaks, or stalled replication forks) can lead to DSBs after replication, which can result in recombination, thus producing permanent genome rearrangements. As shown in yeast, oxidative mutagens might be powerful inducers of DNA deletions (45). Because embryonic cells are generally characterized by a high replication index, they might be particularly susceptible to oxidative genome damage.

We have also observed an inflammatory reaction as shown by changes in cytokine expression in peripheral blood, in which TiO2 nanoparticles could be exerting direct inflammatory effects on circulating innate cells and Th1 effector cells. The inflammatory response involving recruitment and activation of phagocytes is capable of causing oxidative bursts that may serve as a possible explanation for the observed genotoxicity to peripheral leukocytes, micronuclei formation, oxidative DNA damage in liver cells, and DNA deletion induction in fetal RPE. Because we showed that in mice TiO2 nanoparticles induce an inflammatory reaction and oxidative DNA damage, it is tempting to speculate that the mechanism underlying TiO2 nanoparticles genotoxicity might be a secondary (indirect) genotoxicity pathway as suggested by Dankovic and colleagues (3). Secondary genotoxicity is considered to be the key aspect of some particle toxicity (e.g., quartz and silica) because of their ability to elicit persistent inflammation in vivo (9, 46). This implies that particles have prooxidant and proinflammatory activity, leading to genotoxicity.

In summary, our study showed for the first time that TiO2 nanoparticles induce clastogenicity, genotoxicity, oxidative DNA damage, and inflammation in vivo in mice. These results have been observed after only 5 days of treatment via drinking water and in multiple organs suggesting a systemic effect. We also showed that in utero exposure to TiO2 nanoparticles results in an increased frequency in DNA deletions in the fetus. These results represent the first comprehensive in vivo genotoxicity study of TiO2 nanoparticles. These data suggest that we should be concerned about a potential risk of cancer or genetic disorders especially for people occupationally exposed to high concentrations of TiO2 nanoparticles and that it might be prudent to limit ingestion of TiO2 nanoparticles through nonessential drug additives, food colors, etc.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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