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Genotoxicity assessment of titanium dioxide nanoparticle accumulation of 90 days in the liver of gpt delta transgenic mice

Tetsuya Suzuki1,2, Nobuhiko Miura1,3, Rieko Hojo1, Yukie Yanagiba1, Megumi Suda1, Tatsuya Hasegawa4, Muneyuki Miyagawa1,5 and Rui-Sheng Wang1*

Abstract
Background: A variety of in vivo and in vitro studies to assess the genotoxicity of titanium dioxide nanoparticles (TiO2 NPs) have been reported, but the results are inconsistent. Recently, we reported that TiO2 NPs exhibit no genotoxic effects in the liver and erythrocytes during a relatively brief period following intravenous injection into mice. However, there is no information about long-term genotoxicity due to TiO2 NP accumulation in tissues. In this study, we investigated the long-term mutagenic effects of TiO2 NPs and the localization of residual TiO2 NPs in mouse liver after multiple intravenous injections.

Results: Male gpt delta C57BL/6 J mice were administered with various doses of TiO2 NPs weekly for 4 consecutive weeks. The long-term mutagenic effects on the liver were analyzed using gpt and Spi− mutation assays 90 days after the final injection. We also quantified the amount of titanium in the liver using inductively coupled plasma mass spectrometry and observed the localization of TiO2 NPs in the liver using transmission electron microscopy. Although TiO2 NPs were found in the liver cells, the gpt and Spi− mutation frequencies in the liver were not significantly increased by the TiO2 NP administration.

Conclusions: These results clearly show that TiO2 NPs have no mutagenic effects on the liver, even though the particles remain in the liver long-term.

Keywords: Titanium dioxide, Nanoparticles, gpt delta mice, Mutation frequency

Introduction
Titanium dioxide nanoparticles (TiO2 NPs) have become widely used in several industrial applications. Ultrafine TiO2 NPs (10–50 nm) cause lung cancer in rats through chronic inhalation [1]. Therefore, TiO2 NPs are classified as an IARC Group 2B carcinogen (possibly carcinogenic to humans) [1, 2]. Genotoxicity is one of the key factors to assess the carcinogenic risk to humans. Several studies in mice and rats have reported conflicting results of various genotoxic endpoint analyses [3–16]. Recently, we reported that TiO2 NPs have no genotoxic effects in the liver and erythrocytes when intravenously injected into gpt delta mice [17], but there are still reports about their positive effect [18, 19]. Thus, the genotoxicity of TiO2 NPs remains unclear [20, 21].

TiO2 NPs in the rodent bloodstream are translocated to the liver and remain in the tissue long-term [22–25]. However, the long-term genotoxic effects of TiO2 NPs in the liver are not well understood. To investigate the long-term genotoxic effects, the mutagenicity is the optimum endpoint among various genotoxic endpoints because of the accumulation potential of TiO2 NPs. In this study, we examined the long-term mutagenicity of TiO2 NPs and the amount and localization of the remaining particles in the liver after intravenous injection in gpt delta mice [26]. Our results indicated that

* Correspondence: wang@h.jniosh.johas.go.jp
1Division of Industrial Toxicology and Health Effects Research, National Institute of Occupational Safety and Health, 6-21-1 Nagao, Tama-ku, Kawasaki, Kanagawa 214-8585, Japan
Full list of author information is available at the end of the article

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TiO₂ NPs show no mutagenicity in the tissue, although they remain within the liver cells for an extended period of time.

**Materials and methods**

**Animals and reagents**
The guidelines for the care and use of laboratory animals set forth by the Institutional Animal Care and Use Committee of the Japan National Institute of Occupational Safety and Health were followed. Male C57BL/6J gpt delta mice were obtained from Japan SLC (Shizuoka, Japan). They were housed under specific pathogen-free conditions with a 12 h light-dark cycle and provided tap water and sterile CE-2 pellets (CLEA Japan Inc., Tokyo, Japan) ad libitum. Aeroxide® P25 titanium dioxide (TiO₂-P25) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Preparation of the TiO₂ NP suspension and its administration to mice**
The TiO₂-P25 suspension was prepared as previously described [17]. Eight-week-old male gpt delta mice were randomly divided into four groups, with 6 mice per group. Mice were administrated by tail vein injection with the TiO₂-P25 NP suspension at doses of 2, 10, and 50 mg/kg body weight once a week for 4 consecutive weeks. Mice were euthanized on day 90 after the final injection of TiO₂-P25. Portions of the middle liver lobe were removed for gpt and Spi⁻ mutation assays, quantification of titanium by inductively coupled plasma mass spectrometry (ICP-MS), and observation of TiO₂-P25 particles by transmission electron microscopy (TEM).

**gpt and Spi⁻ mutation assay**
The gpt and Spi⁻ mutation assays were conducted as previously described [17].

**Quantification of titanium in the liver by ICP-MS**
Liver samples were weighed and digested with nitric acid and hydrogen peroxide. The concentration of titanium in each sample was measured using ICP-MS (Agilent7900 ICP-MS, Agilent Technologies, Tokyo, Japan). The titanium concentration was determined at the mass number of 47 m/z as previously reported [27].

**Observation of hepatocyte ultrastructure by TEM**
Liver samples taken at day 90 after the final injection of TiO₂-P25 were analyzed using a JEM-2100F transmission electron microscope (JEOL, Tokyo, Japan) as previously described [17].

**Statistical analysis**
Statistical significance was examined using Dunnett’s test after one-way ANOVA. Values of $P < 0.05$ were considered significant.

**Results**

**Characterization of TiO₂ suspensions**
TiO₂-P25 was dispersed in disodium phosphate by sonication, as previously reported [17], then diluted to the concentration corresponding to each dose. The hydrodynamic sizes of TiO₂-P25 in these diluents were measured each time before the injection by dynamic light scattering, and the average of four time determinations was showed in Table 1. The Z-average of the TiO₂-P25 particles in suspension was about 150 d.nm, regardless of the different concentrations in these diluents.

**General observations of the animals**
The body weight of mice in each group did not differ at weekly measurement for the first 4 weeks and thereafter to the end of the experiment (data not shown). However, one mouse was dead immediately after the injection at the third week, but the cause for the death could not be identified. Some of the mice got astounding for a short time immediately after the injection, but the mice in all groups were basically not found with abnormal behaviors and appearance.

**Mutation frequencies of gpt and Spi⁻ in the liver**
The gpt and Spi⁻ mutation frequencies in the liver were determined on day 90 after the last administration of TiO₂-P25 (Tables 2 and 3). Either the gpt or Spi⁻ mutation frequencies were not significantly different between the vehicle control group and TiO₂ administration groups at any dose. These results suggest that TiO₂-P25 has no mutagenic effect on hepatocytes in mice at 90 d after the last administration.

**Quantification and localization of TiO₂ NPs in the liver**
The amount of titanium in the liver of mice administered TiO₂-P25 was quantified via ICP-MS. The average amount of titanium in the liver was dose dependent (Table 4). To clarify the localization of TiO₂ particles, liver sections were obtained from mice treated with 50 mg/kg TiO₂-P25, and the sections were observed by TEM. Large clusters containing the TiO₂ NPs were found in the parenchymal hepatocytes (Fig. 1c) and Kupffer cells (Fig. 1d), although the clusters were much more prevalent in the latter. The particles were extremely agglomerated within the cytoplasm of both cell types. These results indicate that TiO₂ NPs remained in the liver 90 d after the last injection and were mainly localized in the cytoplasm of Kupffer cells.
TiO₂ NPs have been classified as an IARC Group 2B potential carcinogen. Therefore, their genotoxicity is an important property for risk assessment. Several in vivo studies related to genotoxic effects of TiO₂ NPs have been reported, but their results are inconsistent [3–17]. Almost all of these reports analyzed the micronuclei and DNA damage by the comet assay, which reveals transient genotoxic consequences that occur shortly after exposure. In addition, the mutagenicity in TiO₂-accumulating tissues such as the liver and spleen has been examined in transgenic mice for a relatively brief period [8, 17]. Thus, the long-term mutagenic effects of TiO₂ NPs remain unclear. In this study, long-term genotoxic effects were examined in the liver of mice intravenously administered TiO₂ NPs.

Recently, we reported that TiO₂-P25 exhibits no mutagenicity in the liver of mice 9 d after the final injection based on gpt and Spi⁻ mutation assays [17]. However, TiO₂ NPs translocated to the liver are known to accumulate for a long period [22–25]. Thus, we have examined the mutagenicity of TiO₂-P25 in the liver of long-term housed mice after the last administration. TiO₂-P25 caused no mutagenic effects in the liver 90 d after the final injection, even though the particles were observed in the liver cells. The state of the cells that

### Discussion

| Concentration (mg/mL) | Dose (mg/kg b.w.) | Z-average (d.nm) mean ± SD | PdI mean ± SD |
|----------------------|-------------------|---------------------------|--------------|
| 0.4                  | 2                 | 153.6 ± 6.5               | 0.168 ± 0.03 |
| 2                    | 10                | 152.6 ± 7.2               | 0.146 ± 0.01 |
| 10                   | 50                | 148.0 ± 6.0               | 0.172 ± 0.01 |

*d.nm nm of diameter  
*PdI Polydispersity index

| Table 1 | Agglomeration sizes of different concentrations of TiO₂-P25 suspensions in 2 mg/mL disodium phosphate |
|---------|--------------------------------------------------------------------------------------------------|
| TiO₂-P25 Total population Number of mutations Mutation frequency (×10⁻⁶) Mean ± SD         |
| 0 mg/kg 1,005,000 1 1.00                          |
| 1,215,000 5 4.12                          |
| 996,000 2 2.01                          |
| 1,242,000 4 3.22                          |
| 1,074,000 4 3.72                          |
| 1,530,000 5 3.27 2.89 ± 1.17                  |
| 2 mg/kg 1,065,000 7 6.57                          |
| 468,000 8 17.09                          |
| 1,230,000 4 3.25                          |
| 1,089,000 1 0.92                          |
| 1,446,000 4 2.77                          |
| 1,167,000 6 5.14 5.96 ± 5.80                  |
| 10 mg/kg 846,000 3 6.57                          |
| 912,000 5 3.55                          |
| 711,000 2 5.48                          |
| 771,000 4 2.81                          |
| 1,506,000 2 5.19                          |
| 1,380,000 5 1.33 3.66 ± 1.54                  |
| 50 mg/kg 717,000 6 8.37                          |
| 552,000 5 9.06                          |
| 909,000 3 3.30                          |
| 1,011,000 5 4.95                          |
| 1,314,000 4 3.04 5.74 ± 2.82                  |

### Table 2

The gpt mutation frequency in the livers of mice administered TiO₂-P25 90 days after the last administration

| TiO₂-P25 Total population Number of mutations Mutation frequency (x10⁻⁶) Mean ± SD |
|------------------------------|--------------------------|---------------------|
| 0 mg/kg 1,113,000 14 1.26 |
| 1,326,000 20 1.51 |
| 513,000 10 1.95 |
| 1,185,000 13 1.10 |
| 951,000 24 2.52 |
| 1,035,000 14 1.35 1.61 ± 0.53 |
| 2 mg/kg 594,000 16 2.69 |
| 972,000 17 1.75 |
| 855,000 14 1.64 |
| 774,000 13 1.68 |
| 1,344,000 33 2.46 |
| 1,023,000 20 1.96 2.03 ± 0.43 |
| 10 mg/kg 663,000 10 1.51 |
| 738,000 10 1.36 |
| 1,404,000 16 1.14 |
| 1,584,000 17 1.07 |
| 1,440,000 80 5.56 |
| 738,000 11 1.49 2.02 ± 1.74 |
| 50 mg/kg 1,332,000 20 1.50 |
| 345,000 1 0.29 |
| 1,572,000 14 0.89 |
| 1,026,000 17 1.66 |
| 915,000 17 1.86 1.24 ± 0.64 |

### Table 3

The Spi⁻ mutant frequency in the livers of mice administered TiO₂-P25 90 d after the last administration

| TiO₂-P25 Total population Number of mutants Mutant frequency (x10⁻⁵) Mean ± SD |
|------------------------------|--------------------------|---------------------|
| 0 mg/kg 1,113,000 14 1.26 |
| 1,326,000 20 1.51 |
| 513,000 10 1.95 |
| 1,185,000 13 1.10 |
| 951,000 24 2.52 |
| 1,035,000 14 1.35 1.61 ± 0.53 |
| 2 mg/kg 594,000 16 2.69 |
| 972,000 17 1.75 |
| 855,000 14 1.64 |
| 774,000 13 1.68 |
| 1,344,000 33 2.46 |
| 1,023,000 20 1.96 2.03 ± 0.43 |
| 10 mg/kg 663,000 10 1.51 |
| 738,000 10 1.36 |
| 1,404,000 16 1.14 |
| 1,584,000 17 1.07 |
| 1,440,000 80 5.56 |
| 738,000 11 1.49 2.02 ± 1.74 |
| 50 mg/kg 1,332,000 20 1.50 |
| 345,000 1 0.29 |
| 1,572,000 14 0.89 |
| 1,026,000 17 1.66 |
| 915,000 17 1.86 1.24 ± 0.64 |
incorporated TiO\textsubscript{2} NPs after 90 d seemed mostly un-
changed compared to that after 9 d [17]. In addition, the
amount of titanium in the liver at 90 d after the last ad-
ministration was similar to that at 9 d [17]. This result
indicates that TiO\textsubscript{2} NPs are not easily removed from the
liver, even after a long period, but their presence does
not cause any adverse effects.

There were a few in vivo studies reported the positive
genotoxic effect of TiO\textsubscript{2}-NPs. For example, TiO\textsubscript{2} (Aero-
xide P25*) (same material as used in our present study)
induced micronuclei and DNA strand breaks in periph-
eral blood in adult male mice exposed to 500 mg/kg TiO\textsubscript{2} NPs of 21 nm size through drinking water for 5 d
[14], but the effect was not analyzed in liver. With the
same material and intravenous injection route, Dobr-
zyńska et al [4] detected increase of micronuclei in bone
marrow polychromatic erythrocytes of mice only at 24 h
but not later at 7 and 28 d. Modrzynska et al [28] inves-
tigated the DNA strand breaks in the liver of mice
treated with TiO\textsubscript{2} NPs (NanoAmor, 10.5 nm) by intra-
tracheal instillation, intravenous injection or oral gavage
at a single dose of 162 \(\mu\)g/mouse, and did not find DNA
damages in liver tissue on day 1, 28 or 180 after the ex-
posure by any administration routes, though there was a

| TiO\textsubscript{2}-P25 | Analyzed no. of mice | Titanium (\(\mu\)g/g tissue) |
|-------------------|---------------------|-----------------------------|
| 0 mg/kg           | 6                   | 0.10 ± 0.05                 |
| 2 mg/kg           | 6                   | 6.9 ± 4.1                   |
| 10 mg/kg          | 6                   | 16.0 ± 4.0 *                |
| 50 mg/kg          | 5                   | 24.4 ± 9.1 *                |

The data are expressed as mean ± SD
Statistical analysis was conducted by Dunnett’s test; *P < 0.01

**Table 4** The amount of titanium in the livers of mice admini-
stered TiO\textsubscript{2}-P25 90 days after the last administration

![Fig. 1](image) Transmission electron microscope images of mice liver. a and b, parenchymal hepatocyte and phagocyte, respectively, from the liver of control mice, and c and d, parenchymal hepatocyte and phagocyte, respectively, from mice administered with 50 mg/kg TiO\textsubscript{2}-P25. * Photo B was from a control mouse in the short period experiment with TiO\textsubscript{2}-P25 (Ref. no.17)
significant increase in the level of DNA damages in lung tissue on day 180 following intratracheal instillation. These reports suggested that TiO₂ NPs may induce transient DNA damages in tissue such as blood cells, but not in liver. The endpoint of genotoxicity in this study was gene mutations, which are basically not repairable and could indicate the long term effect. The negative findings in this study are supported by many other reports [6–9, 12, 28]. It is known that physicochemical characteristics (primary size, shape, etc.) and study designs (dose, dispersion method, recovery time, models) can influence the toxicity of nanoparticles in the assay system. More studies with different TiO₂ NPs are needed to better understand the health effects of this new material.

Conclusion

TiO₂ NPs accumulate in the liver cells for long term. However, they do not induce genotoxic effect in the tissue. Therefore, the long-term genotoxic effects of TiO₂ NPs administered by inhalation and ingestion which may introduce a small portion of the particles into liver, may be negligible in the liver.

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Authors’ contributions

TS, NM, RH, YY, MS, TH and RSW were involved in data collection. TS and RSW contributed to analyzing the data and drafting the manuscript. MM and RSW contributed to the data interpretation. All authors approved the final manuscript.

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Availability of data and materials

The datasets generated and /or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The animal experiments was approved as stated in the "Animals and reagents".

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

1Division of Industrial Toxicology and Health Effects Research, National Institute of Occupational Safety and Health, 6-21-1 Nagao, Tama-ku, Kawasaki, Kanagawa 214-8585, Japan. Present address: Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima 734-8553, Japan. 2Division of Health Science, Yokohama University of Pharmacy, Yokohama 245-0066, Japan. 3Division of Human Environmental Science, Mount Fuji Research Institute, Yamanashi Prefectural Government, 5597-1 Kenmarubi, Kamiyoshida, Yamanashi 403-0005, Japan. 4Present Address: Department of Sport and Medical Science, Faculty of Medical Technology, Teikyo University, Hachioji, Tokyo 192-0835, Japan.

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