Suzuki et al. Genes and Environment (2020) 42:7
https://doi.org/10.1186/s41021-020-0146-3

Genes and Environment

RESEARCH Open Access

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

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Abstract

Backgound: A variety of in vivo and in vitro studies to assess the genotoxicity of titanium dioxide nanoparticles (TiO_2 NPs) have been reported, but the results are inconsistent. Recently, we reported that TiO_2 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 TiO_2 NP accumulation in tissues. In this study, we investigated the long-term mutagenic effects of TiO_2 NPs and the localization of residual TiO_2 NPs in mouse liver after multiple intravenous injections.

Results: Male *gpt* delta C57BL/6 J mice were administered with various doses of TiO_2 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 TiO_2 NPs in the liver using transmission electron microscopy. Although TiO_2 NPs were found in the liver cells, the *gpt* and Spi^- mutation frequencies in the liver were not significantly increased by the TiO_2 NP administration.

Conclusions: These results clearly show that TiO_2 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 (TiO_2 NPs) have become widely used in several industrial applications. Ultrafine TiO_2 NPs (10–50 nm) cause lung cancer in rats through chronic inhalation [1]. Therefore, TiO_2 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 TiO_2 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 TiO_2 NPs remains unclear [20, 21].

TiO_2 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 TiO_2 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 TiO_2 NPs. In this study, we examined the long-term mutagenicity of TiO_2 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

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TiO_2 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/6 J *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 (TiO_2-P25) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of the TiO_2 NP suspension and its administration to mice

The TiO_2-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 TiO_2-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 TiO_2-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 TiO_2-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 TiO_2-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 TiO_2 suspensions

TiO_2-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 TiO_2-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 TiO_2-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 astounded 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 TiO_2-P25 (Tables 2 and 3). Either the *gpt* or Spi^- mutation frequencies were not significantly different between the vehicle control group and TiO_2 administration groups at any dose. These results suggest that TiO_2-P25 has no mutagenic effect on hepatocytes in mice at 90 d after the last administration.

Quantification and localization of TiO_2 NPs in the liver

The amount of titanium in the liver of mice administered TiO_2-P25 was quantified via ICP-MS. The average amount of titanium in the liver was dose dependent (Table 4). To clarify the localization of TiO_2 particles, liver sections were obtained from mice treated with 50 mg/kg TiO_2-P25, and the sections were observed by TEM. Large clusters containing the TiO_2 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 TiO_2 NPs remained in the liver 90 d after the last injection and were mainly localized in the cytoplasm of Kupffer cells.

Table 1 Agglomeration sizes of different concentrations of TiO_2-P25 suspensions in 2 mg/mL disodium phosphate

Concentration (mg/mL)	Dose (mg/kg b.w.)	Z-average (d.nm[a]) mean ± SD	PdI[b] 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

[a]*d.nm* nm of diameter
[b]*PdI* Polydispersity index

Discussion

TiO_2 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 TiO_2 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 TiO_2-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 TiO_2 NPs remain unclear. In this study, long-term genotoxic effects were examined in the liver of mice intravenously administered TiO_2 NPs.

Recently, we reported that TiO_2-P25 exhibits no mutagenicity in the liver of mice 9 d after the final injection based on *gpt* and Spi^- mutation assays [17]. However, TiO_2 NPs translocated to the liver are known to accumulate for a long period [22–25]. Thus, we have examined the mutagenicity of TiO_2-P25 in the liver of long-term housed mice after the last administration. TiO_2-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

Table 2 The *gpt* mutation frequency in the livers of mice administered TiO_2-P25 90 days after the last administration

TiO_2-P25	Total population	Number of mutations	Mutation frequency ($\times 10^{-6}$)	
				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 3 The Spi^- mutant frequency in the livers of mice administered TiO_2-P25 90 d after the last administration

TiO_2-P25	Total population	Number of mutants	Mutant frequency ($\times 10^{-5}$)	
				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

Table 4 The amount of titanium in the livers of mice administered TiO_2-P25 90 days after the last administration

TiO_2-P25	Analyzed no. of mice	Titanium (μg/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; *$P < 0.01$

incorporated TiO_2 NPs after 90 d seemed mostly unchanged compared to that after 9 d [17]. In addition, the amount of titanium in the liver at 90 d after the last administration was similar to that at 9 d [17]. This result indicates that TiO_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_2-NPs. For example, TiO_2 (Aeroxide P25®) (same material as used in our present study) induced micronuclei and DNA strand breaks in peripheral blood in adult male mice exposed to 500 mg/kg TiO_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, Dobrzynska 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] investigated the DNA strand breaks in the liver of mice treated with TiO_2 NPs (NanoAmor, 10.5 nm) by intratracheal instillation, intravenous injection or oral gavage at a single dose of 162 μg/mouse, and did not find DNA damages in liver tissue on day 1, 28 or 180 after the exposure by any administration routes, though there was a

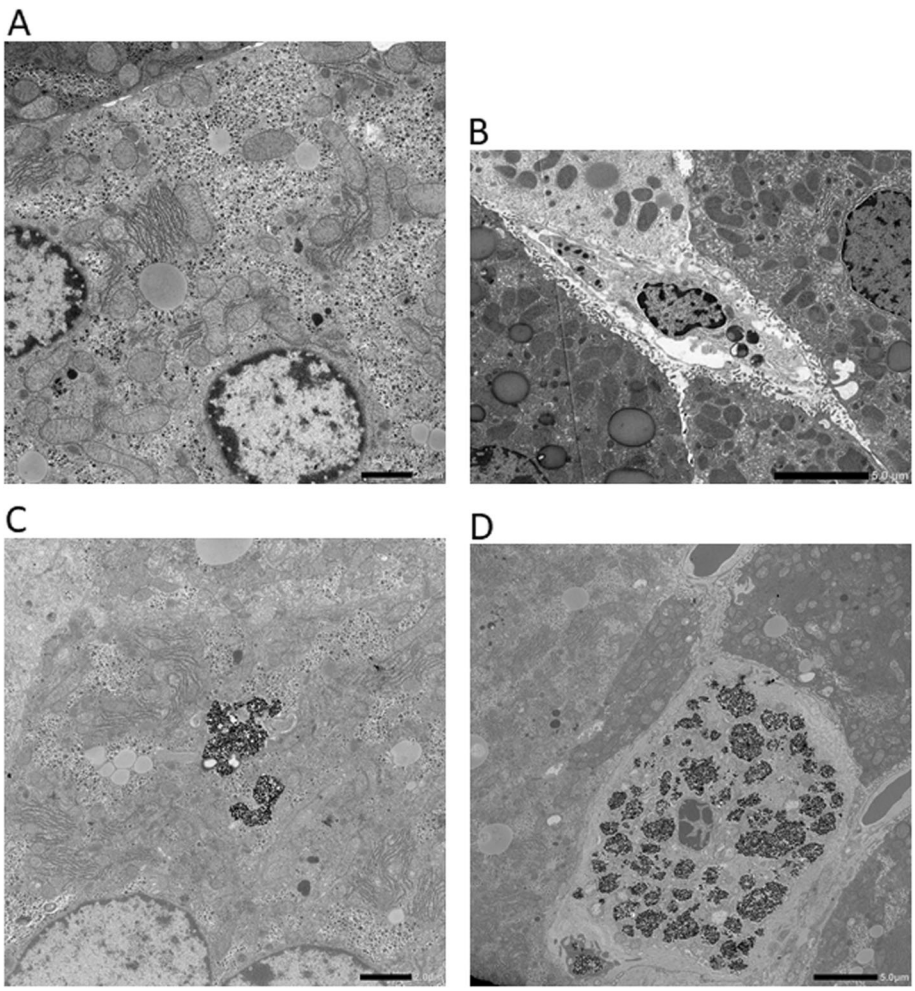

Fig. 1 Transmission electron microscope images of mice liver. **a** and **b**, parenchymal hepatocyte and phagocyte, respectively, from the liver of control mice, and **c** and **d**, parenchymal hepatocyte and phagocyte, respectively, from mice administered with 50 mg/kg TiO_2-P25. * Photo B was from a control mouse in the short period experiment with TiO_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 TiO_2 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 TiO_2 NPs are needed to better understand the health effects of this new material.

Conclusion

TiO_2 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 TiO_2 NPs administered by inhalation and ingestion which may introduce a small portion of the particles into liver, may be negligible in the liver.

Acknowledgements

We are deeply grateful to Dr. Masumura, Division of Genetics and Mutagenesis, National Institute of Health Sciences, Kawasaki, Japan, for providing the *E. coli* strains for *gpt* and Spi$^-$ mutation assays.

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.

Funding

This study was funded by the National Institute of Occupational Safety and Health, Japan (N-P24–02).

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.

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Received: 24 October 2019 Accepted: 27 January 2020
Published online: 10 February 2020

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