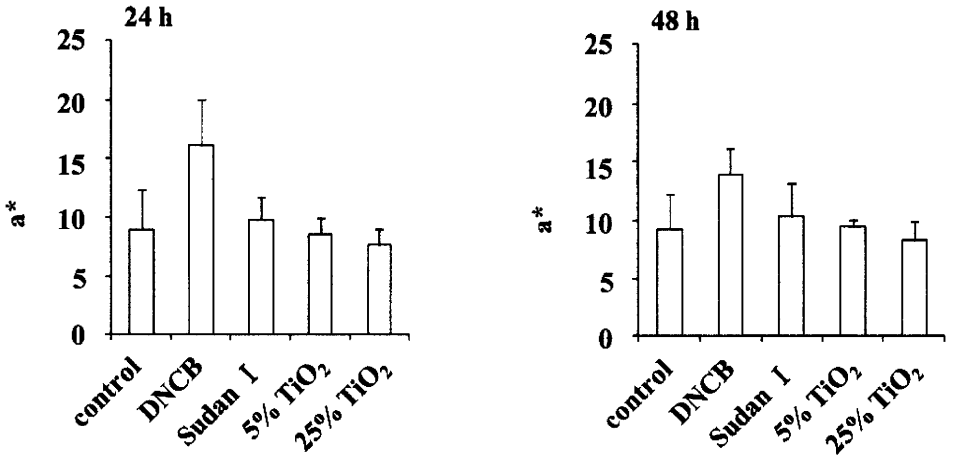


Fig. 14 Effect of non-coated rutile type TiO_2 particles exposure on the a* value.
Values are the mean ± S.E. (n = 4).

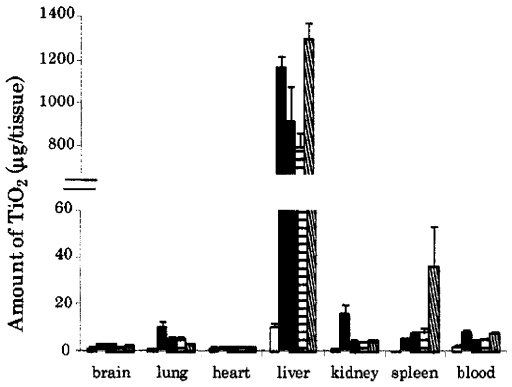


Fig. 15 Amount of TiO_2 in several tissues after *i.v.* injection of silica coated TiO_2 particles.
Symbols:□: control, ■: 5 min, ■ : 72 h, ■ : 1 month, ■ : 6 months.
Values are the mean ± S.E. (n = 3-5).

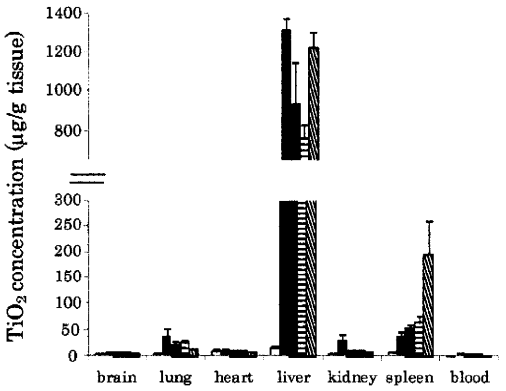
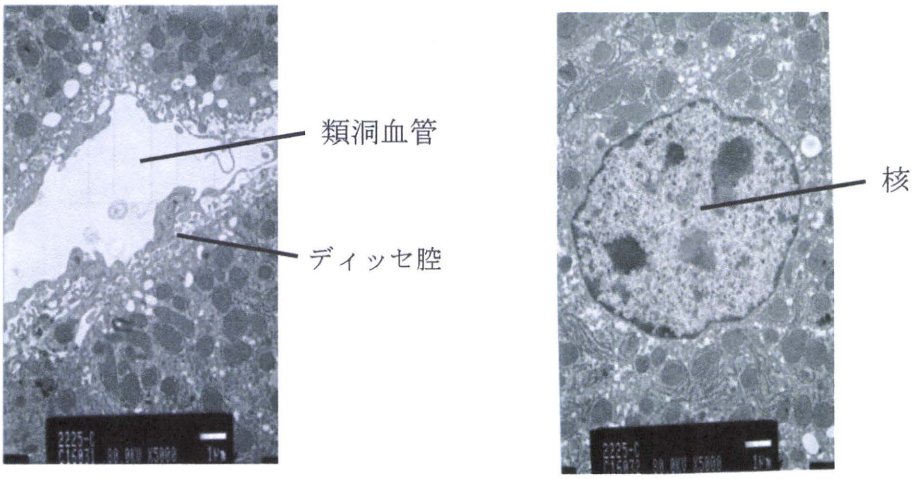
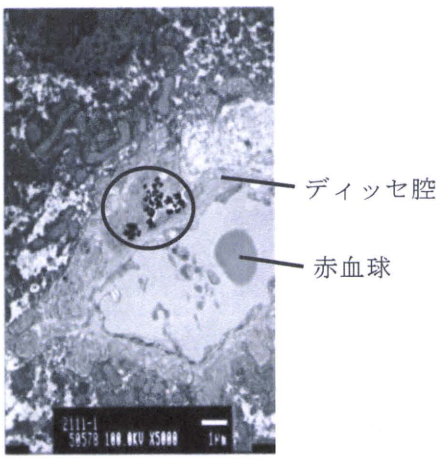


Fig. 16 Concentrations of TiO_2 in several tissues after *i.v.* injection of TiO_2 particles.
Symbols:□: control, ■: 5 min, ■ : 72 h, ■ : 1 month, ■ : 6 months.
Values are the mean ± S.E. (n = 3-5).

(a) Control



(b) 72 h after *i.v.* injection



(c) 1 month after *i.v.* injection

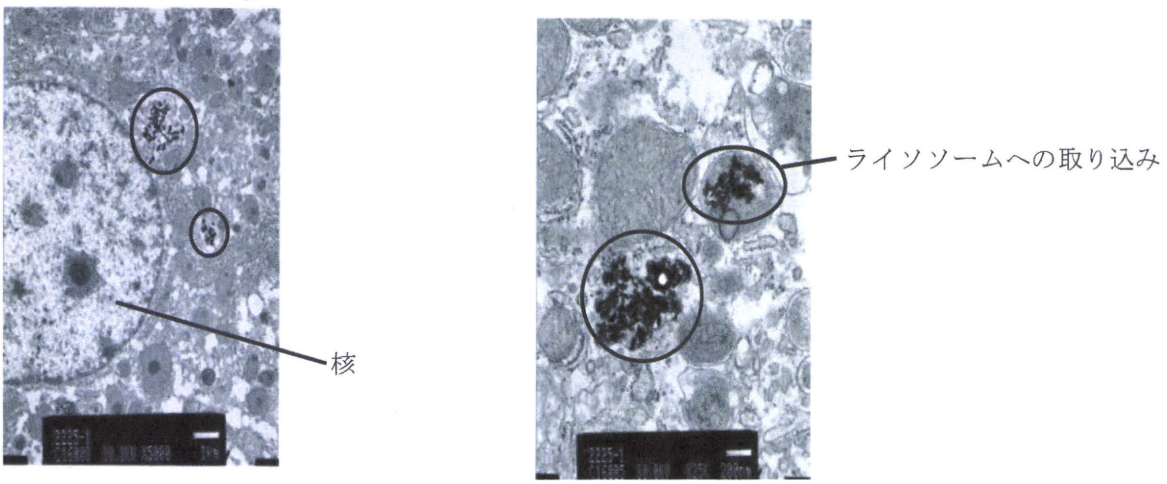


Fig. 17　TEM images on mouse liver after *i.v.* injection of TiO_2 particles.
(a): control, (b): 72 h after *i.v.* injection, (c): 1 month after *i.v.* injection.

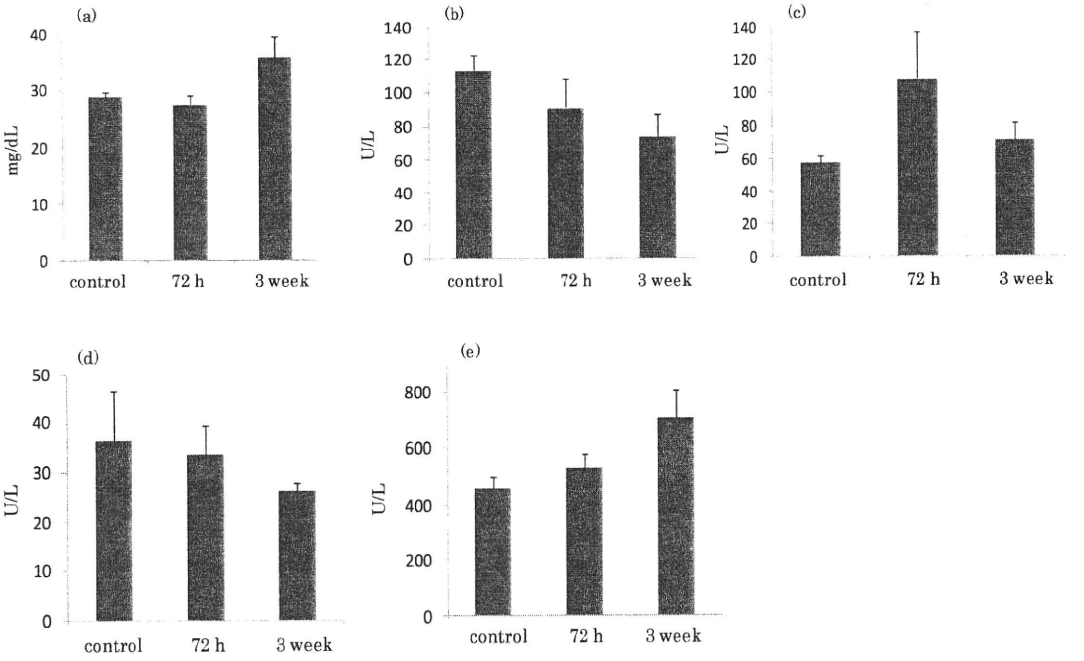


Fig. 18 Effect of TiO_2 nanoparticles on the inflammatory markers in mice.
(a) : BUN, (b) : CPK, (c) : AST, (d) : ALT, (e) : LDH. Values are the mean ± S.E. (n = 5).

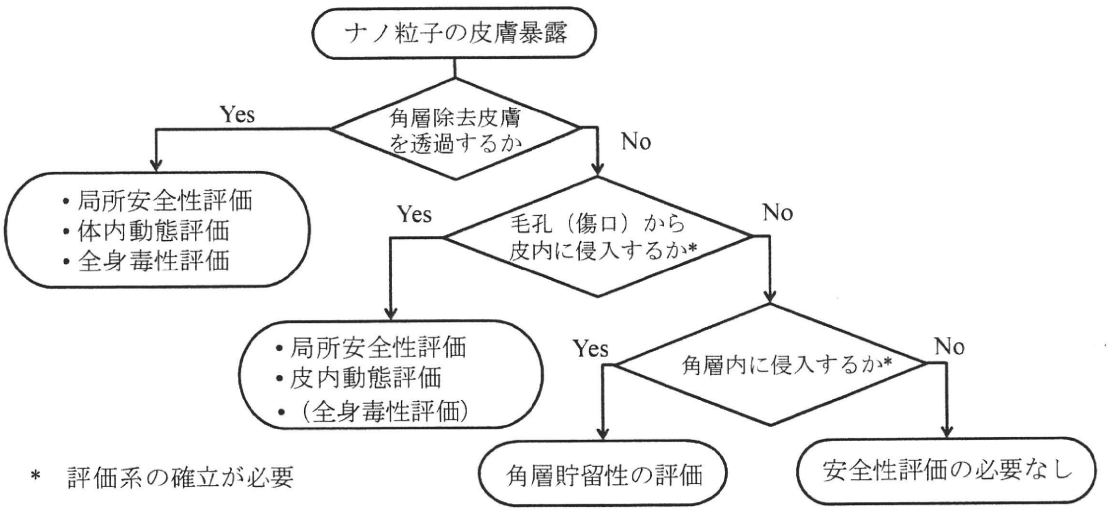


Fig. 19 Scheme of evaluation methods for skin exposure of nanoparticles.

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Senzui M, Tamura T, Miura K, Ikarashi Y, Watanabe Y, Fujii M.	Study on penetration of titanium dioxide (TiO_2) nanoparticles into intact and damaged skin in vitro	J. Toxicol. Sci.	35 (1)	107-113	2010
Uchino, T., Ikarashi, Y., Nishimura, T.	Effects of coating materials and size of titanium dioxide particles on their cytotoxicity and penetration into the cellular membrane	J. Toxicol. Sci.	36 (1)	95-100	2011
K. Sugibayashi, H. Todo, E. Kimura	Safety evaluation of titanium dioxide nanoparticles by their absorption and elimination profiles	J. Toxicol. Sci.	33 (3)	293-298	2008
杉林堅次	紫外線防御試験法の国際的動向と紫外線防御剤の開発の課題：化粧品に用いられるナノ粒子の曝露と安全性問題のあり方	FRAGRANCE JOURNAL	36 (10)	38-41	2008
Todo H., Kimura E., Yasuno H., Tokudome Y., Hashimoto F, Ikarashi Y., Sugibayashi K.	Permeation pathway of macromolecules and nanospheres through skin	Biol. Pharm. Bull.	33 (8)	1394-1399	2010

IV.　研究成果の刊行物・別刷

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Letter

Study on penetration of titanium dioxide (TiO_2) nanoparticles into intact and damaged skin *in vitro*

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ABSTRACT — It is important for toxicological assessment of nanoparticles to determine the penetration of nanoparticle in skin qualitatively and quantitatively. Skin penetration of four different types of rutile titanium dioxide (TiO_2) (T-35, 35 nm, non-coating; TC-35, 35 nm, with almina/silica/silicon coating; T-disp, 10 x 100 nm, mixture of almina coated and silicon coated particles, dispersed in cyclopentasiloxan; T-250, 250 nm, non-coating) was determined with *in vitro* intact, stripped, and hair-removed skin of Yucatan micropigs to study the effect of dispersion and skin conditions. The TiO_2 was suspended in a volatile silicone fluid used for cosmetics, cyclopentasiloxane, at a concentration of 10%. The suspension was applied at a dose 2 μl/cm^2 for 24 hr, followed by cyanoacrylate stripping. The Ti concentration in skin was determined by ICP-MS. T-35 and T-250 easily aggregated in suspension with a mean diameter greater than 1 μm. TC-35 and T-disp showed good dispersion properties with a mean diameter in suspension of approximately 100 nm. No penetration was observed regardless of TiO_2 type in intact and stripped skin. The concentration of Ti in skin was significantly higher when TC-35 was applied on hair-removed skin. SEM-EDS observation showed that Ti penetrated into vacant hair follicles (greater than 1 mm below the skin surface), however, it did not penetrate into dermis or viable epidermis.

Key words: Nanoparticle, Skin penetration, Hair-removed skin, Stripped skin, Titanium oxide

INTRODUCTION

Titanium dioxide (TiO_2) has been used for cosmetics and is considered to be safe for topical use. Recently, TiO_2 nanoparticles (particle size < 100 nm) are used commonly in cosmetics because of their high transparency in visible wavelengths but high attenuation for UV wavelengths (Popov *et al.*, 2005). However, the safety of different conventional size particles is a concern. Safety concerns are based on not only its toxicity characteristics, but also on the potential increase in amount of absorption. In theoretical, materials with an appropriate octanol/water partition coefficient and low molecular weight (< *ca.* 500) penetrate skin through the stratum corneum (SC); therefore, penetration of inorganic particles into intact skin is not possible (Roberts *et al.*, 2002). Some studies indicate that TiO_2 and other inorganic particles, even on a nano-grade scale, do not penetrate skin (Schulz *et al.*, 2002; Pinheiro *et al.*, 2007; Nohynek *et al.*, 2008). However, some nanoparticles can penetrate viable skin (Ryman-Rasmussen *et al.*, 2006; Menzel *et al.*, 2004). Inorganic particles are often lyophobic in both water and oil, dispersed particles easily aggregate to form large (micro-grade) particles. A few studies investigating both dispersibility and skin penetration have been reported (Bennat and Müller-Goymann, 2000). The present study focused on skin penetration of TiO_2 *in vitro* with different dispersibility of TiO_2 and skin condition.

MATERIALS AND METHODS

Materials

All types of TiO_2 used in this study were rutile-type (Table 1). Cyclopentasiloxane (silicone, KF-995, Shin-Etsu Chemical, Co., Tokyo, Japan) was used as the dispersing medium. Purified water and nitric acid used in TiO_2 quantitative analysis were ultra-microanalysis grade from Wako Pure Chemicals Industries, Ltd. (Osaka,

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Table 1. Titanium dioxide used in this study

Abbreviation	Primary particle size*	Coating
T-35	35 nm	uncoated
TC-35	35 nm	alumina · silica · silicone
T-disp	10 nm x 100 nm	mixture of alumina coated and silicone coated
T-250	250 nm	uncoated

All TiO_2 are rutil-type.
* from catalogue of source company.

Japan). A stock solution of titanium containing 1,000 mg/l (Kanto Chemical Co., Inc., Tokyo, Japan) was used to produce standards for calibration curves for Ti analysis. All other chemicals were of reagent grade.

Preparation of suspensions

Drops of silicone were added to a weighed amount of TiO_2 powder in a tube, followed by kneading. Additional silicone was added to bring the concentration of TiO_2 samples to 10%, followed by sonication in a bath-type sonicator (US-3, Iuchi, Tokyo, Japan) for 30 min. The T-disp was diluted with silicone for a final TiO_2 concentration of 10%, followed by sonication.

Evaluation of TiO_2 suspensions

The particle size of TiO_2 in suspension was measured using a dynamic laser scattering apparatus (DLS-8000HL, Otsuka Electrons Co., Osaka, Japan) after a thousand-fold dilution with silicone.

Skin conditions after application of TiO_2 was observed using two methods. Two µl of suspension were applied to an area of skin of approximately 1 cm^2. After drying, the skin surface was observed by digital fine scope microscopy (VC-3000, Omron, Tokyo, Japan) with a magnification of 80. The epidermis of YMP skin prepared by a heat separating method (Kligman and Christophers, 1963) was mounted on a scanning electron microscopy (SEM) stage with adhesive tape. One µl of the suspension were spread over approximately 0.5 cm^2 and dried *in vacuo*. Then, the skin sample was coated with Pt/Pd and examined using SEM (JSM-5200LV, JEOL, 20 kV).

Skin penetration

Yucatan micropig (YMP) skin was used as the model because of its similarity with human skin (Lavker *et al.*, 1991; Fujii *et al.*, 1997). YMP skin (YMP skin set, Charles River Japan, Kanagawa, Japan) removed the subdermal tissue and fat was used as full-thickness skin (intact skin). The SC was removed from intact skin with adhesive tape (Scotch 313, 3M) (stripped skin). Hair was removed from intact skin using tweezers (hair-removed skin).

Two µl of suspension were applied to an area of skin of approximately 1 cm^2. Then the skin was placed on a modified Franz-type diffusion cell. After 24 hr, the receptor phase (pH 7.1 isotonic phosphate buffer solution) was collected, the skin was removed from the diffusion cell and cut off the rim for mounting the cell. Residues on the skin surface were removed by two cyanoacrylate (Aronalfa, Toagosei, Tokyo, Japan) stripping and Ti in the skin was determined. Application amount and period were in accordance with Standard SPF Test Method of Japan Cosmetic Industry Association (1999) and OECD (2004): Test Guideline 428 (skin absorption: *in vitro* method), respectively. For some samples, the epidermis and dermis were separated by heating after cyanoacrylate stripping. A similar procedure was used for obtaining SEM pictures with energy dispersed X-ray spectrometry (SEM-EDS).

Determination of Ti

Approximately 0.1 g of skin or 1 ml of receptor phase was transferred to a Teflon digestion vessel and 5 ml of nitric acid plus 1 ml of purified water was added to each vessel. The vessels were placed in a microwave oven (MARS5, CEM Co., Matthews, NC, USA). The microwave-assisted digestion consisted of increasing the pressure to 80 psi over 20 min and then maintaining that pressure for 20 min by applying 100% power at 1,600 W. For separated epidermis and dermis, approximately 1 cm^2 of skin (*ca.* 0.01 g of epidermis, 0.3 g of dermis) was used. After digestion, the resulting solution was fixed with 20 ml of purified water. The Ti concentration in the samples was measured by ICP-MS (7500, Agilent Technologies, Santa Clara, CA, USA). The amount of Ti was calculated using a standard curve of Ti created with the peak area at mass number 47.

Ti distribution in skin

The skin sample was fixed with Karnovsky solution, dehydrated with ethanol, and replaced with resin. Horizontal cuts were made in the skin from the surface to the dermis and observations were obtained every 50 μm using SEM-EDS (JSM-6700/JED2300, JEOL, Tokyo, Japan).

Statistical analysis

The amount of Ti in skin was determined using at least 3 samples and the data subjected to analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Particle size of TiO_2 in suspension and on the skin surface

Both oil in water (O/W) and water in oil (W/O) creams are used as sunscreen formulations with TiO_2. TiO_2 is usually formulated in the oil phase; the oil suspension using silicone, which is often used for the base of sunscreens because of volatile and repels water characteristics, was used for this study. Many types of TiO_2 particles exist, with differences in crystalline type, size, shape, and surface coating characteristics. Four types of rutile TiO_2 shown in Table 1 was used for this study. T-35 was used to represent typical nanoparticles, with a round shape and no surface coating. TC-35, which has lipophilic coating features, was chosen to produce a good dispersion in silicone. T-disp is a pre-formulated product consisting of TiO_2 dispersed in silicone. The T-250 was used for comparison because it does not form nanoparticles.

Even if the primary particle size is less than 100 nm, lyophobic colloidal dispersions are unstable and aggregation occurs easily. Particle size distributions of TiO_2 in silicone are shown in Fig. 1. Mean particle size of T-35 was 1,700 nm, which was larger than that of T-250, 1,200 nm. Although the possibility exists that large particles shielded small particles, few nanoparticles were found. In contrast, suspensions of TC-35 and T-disp contained nanoparticles with mean diameters of 80 and 130 nm, respectively. The TC-35 suspension contained large particles that were easy to precipitate.

After skin application of suspensions, silicone was spread and vaporized so that only TiO_2 particles remained on the skin. Fig. 2(a) shows microscopic pictures of the surface after application of each suspension to skin followed by drying. T-250 and T-35 suspension showed aggregated white powder. After application of the TC-35 suspension, the skin was covered with white film that was thicker in furrows. Silicone spread easily on the skin and tended to collect in furrows because of low viscosity and interface tension. No particles were observed but the skin was slightly white after application of T-disp suspension. The SEM pictures showed large agglomerated TiO_2 (about 5 μm) for T-35 and T-250, although their primary particle sizes were different. The TC-35 also aggregated into particles of approximately 1 μm, although many small particles stuck to the skin. The T-disp formed uniformly agglomerated particles that differed from other preparations. TiO_2 particles appeared to be covered with dispersing agent (Figs. 2(b), (c)).

Skin penetration of TiO_2

Cosmetics and sunscreens are usually used only on intact skin. However, skin can be injured slightly by objects or through physical force. Thus, skin penetration of TiO_2 was investigated *in vitro* with intact skin and with stripped skin as a model of injured skin. Previous reports have indicated that hair follicles are important in the skin penetration of nanoparticles (Lekki *et al.*, 2007; Zvyagin *et al.*, 2008). Therefore, hair-removed skin was used to represent skin damaged by hair-removal treatments often done for the pursuit of beauty.

After 24-hr application, the skin surface was stripped with cyanoacrylate to remove surface TiO_2. A tape stripping technique is often used to remove residual materials on skin; however, materials in furrows or on hair follicles cannot be removed by this technique (Pflücker *et al.*, 1999). In this study, the amount of Ti varied greatly when

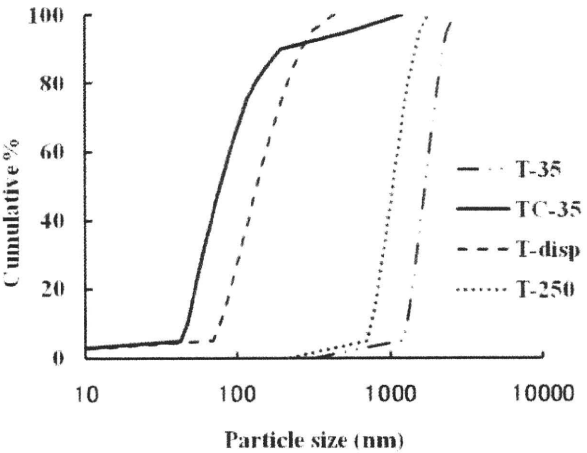

Fig. 1. Particle size of TiO_2 dispersed in silicone. Particle size was measured by dynamic laser scattering using a thousand-fold dilution of 10% TiO_2 silicone suspension.

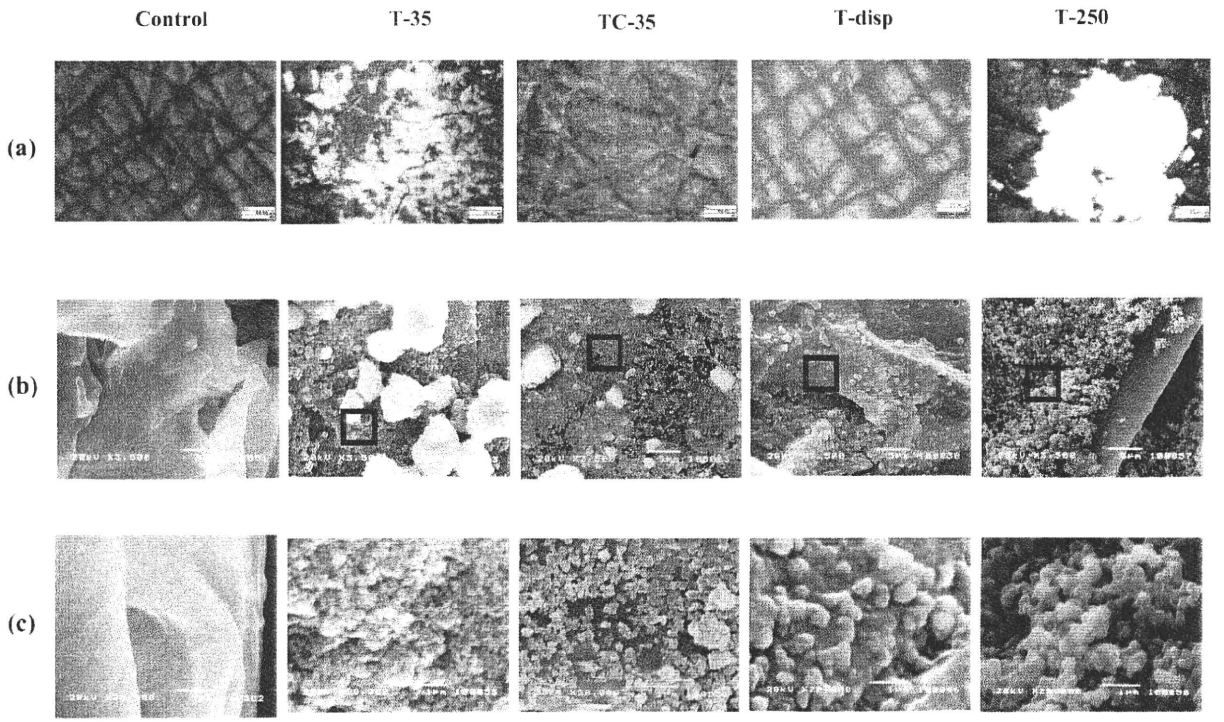

Fig. 2. The conditions of TiO_2 after skin application of a 10% silicon suspension of TiO_2 at a dose of 2 μl/cm^2. The skin surface was observed using a digital fine scope at a magnification of (a) 80. The surface of the epidermis was observed by SEM at a magnification of (b) 3,500 and (c) 20,000.

Ti remained in furrows (data not shown), so cyanoacrylate stripping was used. In this procedure, the surface SC layer of intact skin and hair-removed skin, and some hairs of intact skin and stripped skin were also removed.

Silicone is used to control formulation properties. Ti concentration in the receptor phase was similar in all skin conditions and formulations applied (Fig. 3(a)). Fig. 3(b) shows Ti concentration values in skin. For intact and stripped skin, no significant difference in Ti concentration was found between the control and suspension applications, which indicates TiO_2 did not penetrated into the skin regardless of particle size and even when the SC, which is the skin's primary barrier, was removed. For hair-removed skin, Ti concentration in skin after application of TC-35 suspension was significantly higher than that of the control, and after application of T-disp suspension, tended to be high. The Ti concentration in the dermis was no different than that of the control. Ti concentration in the epidermis after application of TiO_2 nanoparticles tended to be greater than that of the control, but the difference was not significant (Fig. 4). The epidermis consists of SC, viable epidermis and hair follicles. The horizontal sections from hair-removed skin after application of TC-35 suspension were observed using SEM-EDS. One of two SEM-EDS images showed the presence of Ti in the empty hair follicle after removal of the hair shaft 1 mm from the surface (Fig. 5(a)). Ti was detected in the hair follicle pocket, but not in the surrounding viable skin (Fig. 5(b)). We also found the similar distribution of 20 nm FITC-polystyrene (data not shown). The radius of a hair follicle is 0.05-0.2 mm (Otberg *et al.*, 2004), which would allow solvent to enter if the hair shaft and sebum did not fill the follicle space. When fluid enters a small space by capillary action, small particles of Ti in fluid may be able to enter the follicle. Large particles cannot be moved by such a small force, but TC-35 well dispersed in solvent might enter a follicle more easily than other types of TiO_2. For T-disp, the dispersing agent had some effect, resulting in particles left on the skin after drying of the suspension.

In conclusion, TiO_2 does not penetrate into viable skin, even if the particle size is less than 100 nm and the SC is damaged. However, immediately after hair removal, some TiO_2 particles penetrated relatively deeply into the

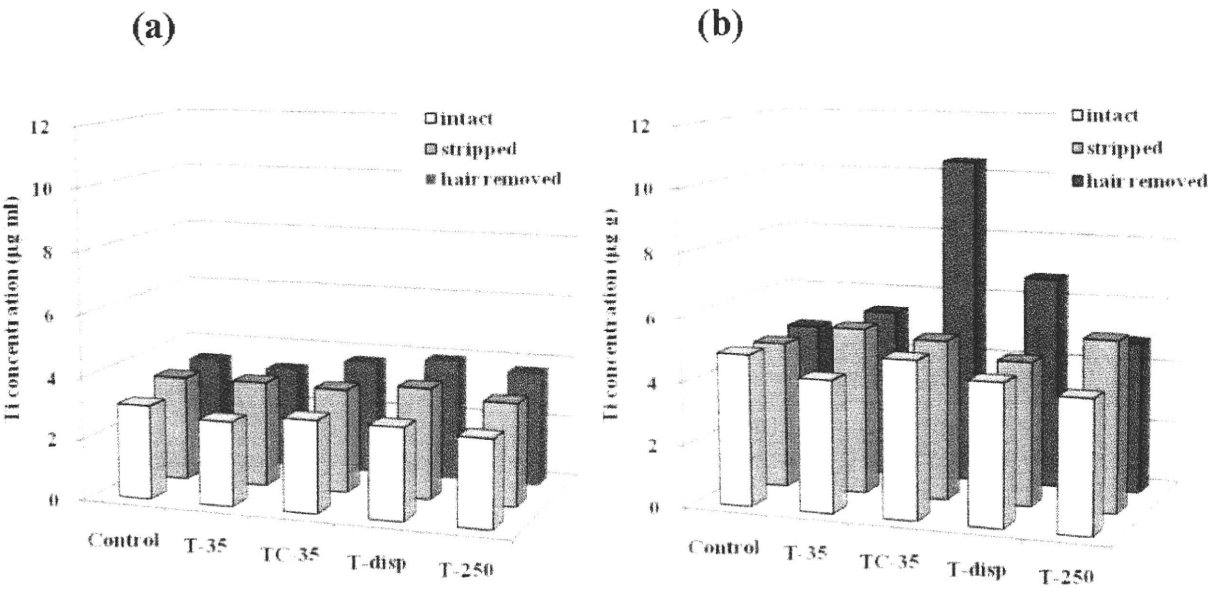

Fig. 3. Ti concentration in receptor phase (a) and in skin (b) after 24 hr application of a 10% silicone suspension of TiO_2 at a dose of 2 μl/cm² on intact skin, stripped skin, and hair-removed skin.
Silicon applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping.

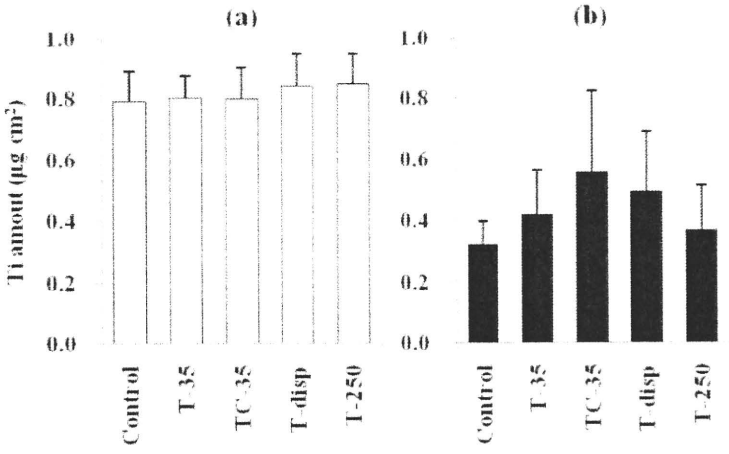

Fig. 4. Amount of Ti in (a) dermis and (b) epidermis after 24 hr application of a 10% silicone suspension TiO_2 at a dose of 2 μl/cm² to hair-removed skin.
Silicone was applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping, followed by separation of the dermis and epidermis by heat.

skin, possibly by entering the empty hair follicle. This was an *in vitro* study, so no inflammation was induced by the hair removal procedure. Inflammation could affect these results, therefore, further *in vivo* studies on viable skin with the hair removed are necessary.

There might be various nanoparticls to determine skin penetration, thus *in vitro* method is important for screening. If the materials have possibility to use on hair

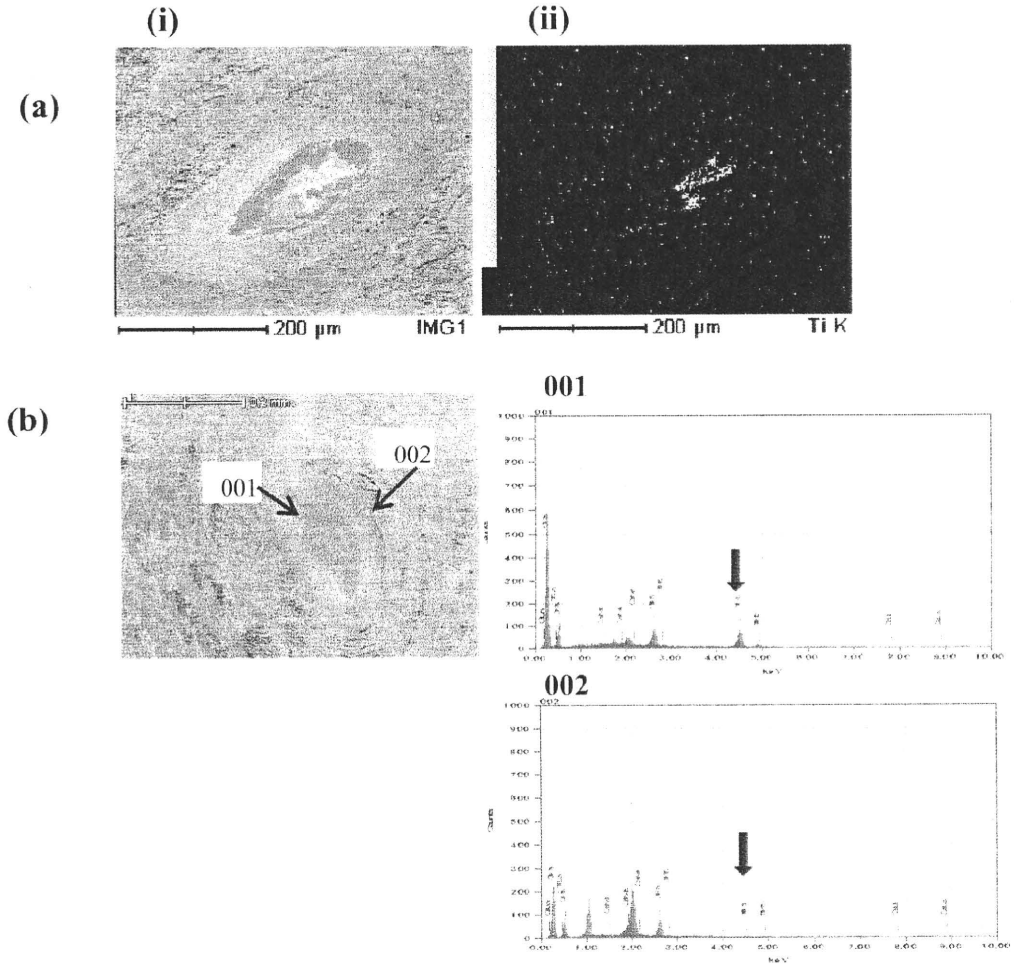

Fig. 5. SEM-EDS images (a) and elemental analysis (b) of a horizontal section of skin after 24 hr application of a 10% silicone suspension of TC-35 at a dose of 2 μl/cm² to hair-removed skin.
(a) at a depth of 1,050 μm from the skin surface, (i) SEM images, (ii) Ti distribution; (b) at a depth of 1,250 μm from the skin surface, (001) the hair follicle, (002) the dermis in contact with the hair follicle.

removed skin, hair removed skin should be taken into safety assessment of nanoparticles as well as stripped skin as a model of damaged skin. Also, this results indicate that the split skin (thickness 200~400 μm) which OECD Test guideline 428 recommended for skin absorption study *in vitro* have possibility to overestimate the skin permeation of nanoparticles because hair follicle is cut and nanoparticle in hair follicle is into receptor phase.

ACKNOWLEDGMENTS

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Letter

Effects of coating materials and size of titanium dioxide particles on their cytotoxicity and penetration into the cellular membrane

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ABSTRACT — In order to estimate the effects of the size and surface treatment (coating or non-coating) of titanium dioxide particles on their cytotoxicity and penetration into the cellular membrane, two types of non-treated titanium dioxide (TiO_2) particles of 20 nm (LU175) and 250 nm (LU205) were exposed to CHO cells, RBL-2H3 cells, A431 cells, B16 melanoma, NHEK(F), and NHSF, and six types of surface-treated or non-treated TiO_2 particles of 35 nm were exposed to RBL-2H3 cells and NHSF. The order of half-maximal inhibitory concentrations (IC50s) of LU175 was NHSF < CHO, RBL-2H3 < A431 < B16 melanoma, NHEK(F). On the other hand, LU205 showed no cytotoxicity against any cells. Surface-treated TiO_2 showed much less cytotoxicity against RBL-2H3 cells than non-treated TiO_2. Then, between 0.5 and 10 mg of LU175 or LU205 was exposed to CHO cells. After 24 hr, the amount of LU175 in cellular cytosol increased dose-dependently. On the other hand, the amount of LU205 in cellular cytosol was much less than that of LU175. The proportion of surface-treated TiO_2 in the cellular cytosol of RBL-2H3 cells differed for each coating material. These results suggested that TiO_2 has different cytotoxicities among cell lines, and that of surface-treated TiO_2 was weaker than that of non-treated TiO_2. TiO_2 located in cytosol might be the main cause of cytotoxicity.

Key words: Nanomaterials, Titanium dioxide, Cytotoxicity, Penetration, RBL-2H3 cells, NHSF

INTRODUCTION

In recent years, the use of nanomaterials such as titanium dioxide (TiO_2) in cosmetics such as sunscreen has advanced rapidly without adequate evaluation of their safety (Ishii, 2005; Hatakeyama, 2005; Lovern and Klaper, 2006; Xia *et al.*, 2006). Hussain *et al.* (2005) demonstrated that TiO_2 (40 nm) at higher doses (100-250 μg/ml) did not induce significant cytotoxicity against BRL 3A rat liver cells, while silver nanoparticles at lower doses (10-50 μg/ml) induced significant cytotoxicity against the cells. Reeves *et al.* (2008) demonstrated that TiO_2 (anatase, 5 nm) at 1,000 μg/ml induced slight cytotoxicity against goldfish skin cells without Ultraviolet-A(UVA)-irradiation. On the other hand, Sayes *et al.* (2006) demonstrated that nanoscale TiO_2 at 100 μg/ml induced significant cytotoxicity and inflammation against human dermal fibroblasts and human lung epithelial cells. Onuma *et al.* (2009) demonstrated that nano-sized TiO_2 had strong cytotoxicity against mouse fibrosarcoma cells. However, few reports about cytotoxicity of nano-sized TiO_2. In particular, little is known about cytotoxicity of nano-sized TiO_2 against various kinds of cells. Thus, it was considered necessary to compare cytotoxicity of nano-sized TiO_2 among various kinds of cells to estimate the potential toxicity of TiO_2. In order to estimate the toxicity of TiO_2, we examined the cytotoxicity of two kinds of non-coating rutile form TiO_2 with particle sizes of 20 nm (LU175) and 250 nm (LU205) against various kinds of cells and transferred LU175 and LU205 into the cellular components.

Surfaces of TiO_2 are usually coated with SiO_2, $Al(OH)_3$, Al_2O_3, or silicone for use in cosmetics. Warheit *et al.* (2007) demonstrated that surface-treated material affects TiO_2 toxicity against rat lung. However, there are few reports investigating the cytotoxic effects of coating materials on cells and the penetration of TiO_2 into cellular components. We examined the cytotoxicity of surface-treated and non-treated TiO_2 against cultured cells and

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transferred TiO_2 into the cellular components.

MATERIALS AND METHODS

Cells

C57BL/6 mouse melanoma (B16 melanoma), Chinese hamster ovary (CHO) cells, and rat basophilic leukemia (RBL-2H3) cells were purchased from Health Science Research Resources Bank (Osaka, Japan). Normal human skin fibroblasts (NHSF) and human epidermoid carcinoma (A431) cells were purchased from Riken Bioresources Center Cell Bank (Ibaraki, Japan). Normal human epidermis keratinocytes from neonatal foreskin (NHEK(F)) were purchased from Kurabo Industries Ltd. (Osaka, Japan).

Culture medium

Eagle's-MEM containing 10% FBS was used as culture medium for B16 melanomas, CHO cells and RBL-2H3 cells. Epi-Life KG2 was used as culture medium for A431 cells and NHEK(F). α-MEM containing 10% FBS was used as culture medium for NHSF.

Particles

Non-coating materials

LU175 and LU205 were obtained from Isihara Sangyo Kaisha, Ltd. (Osaka, Japan). TiO_2 (rutile form) particles of 35 nm (MT-500B; TiO_2 96%) were obtained from Teika Co. (Osaka, Japan).

Coating materials

TiO_2 (rutile form) particles of 35 nm coated with $Al(OH)_3$ and stearic acid (TTO-55(C); TiO_2 90%) and such particles coated with $Al(OH)_3$ (TTO-55(A); TiO_2 96%) were obtained from Isihara Sangyo Kaisha, Ltd. TiO_2 (rutile form) particles of 35 nm coated with $Al(OH)_3$, SiO_2, and silicone (SMT-500SAS; TiO_2 80%), such particles coated with $Al(OH)_3$ and SiO_2 (MT-500SA; TiO_2 85%), and such particles coated with Al_2O_3 (MT-500H; TiO_2 90%) were obtained from Teika Co.

Microwave digestion

After collecting cellular membrane, microsome, and cytosol, these components were added to teflon vessels, and then 5 ml of HNO_3 was added to each vessel. Then, the vessel contents were digested for 22 min (up to 80 PSI for 20 min, maintained for 2 min) using a microwave (MARS 5, CEM Co., Matthews, NC, USA).

ICP-MS measurement

The digested solution was diluted 5 times with milli-Q water and Ti contents were determined by ICP-MS (HP-4500, Hewlett-Packard Co., Palo Alto, CA, USA). ICP-MS conditions were as follows:

RF power:1450 W, RF refraction current: 5 W

Monitoring mass: m/z 48 (Ti)

Cytotoxicity

B16 melanomas, CHO cells, and RBL-2H3 cells were suspended in 0.1 ml of culture medium at a concentration of 5×10^4 cells/ml. A431 cells and NHEK(F) were suspended in 0.1 ml of culture medium at concentrations of 4×10^4 cells/ml and 5×10^4 cells/ml, respectively. NHSF was suspended in 0.1 ml of culture medium at a concentration of 4×10^4 cells/ml. All cells were seeded in a 96-well microplate and cultured for 2 days for CHO cells, 5 days for A431 cells, or 3 days for other cells under 5% CO_2 at 37°C.

TiO_2 particles were suspened in culture medium and ultrasonicated for 5 min. Immediately after ultrasonication, culture medium in the each microplate were removed and suspension (0.1 ml) of LU175 or LU205, the concentration of which ranged from 0 to 10 mg/ml, was added to each well. After 24 hr, cells were washed 5 times with medium and then for the viability assay, 0.1 ml of tetra-color one dissolved in medium was added, followed by incubation for 2 hr. Tetra -color one is a mixture of water-soluable tetrazolium solt [2-(2-Methoxy-4-nitrophenyl)-3-(4-nitorophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)] and electron carrier (1-Methoxy-5-methylphenazinium methylsulfate) converted to a water-soluable formazan by dehydrogenase enzymes located mainly in the mitochondria of living cells. Absorbance at 490 nm (reference at 650 nm) was measured with a microplate reader (Spectramax M5, Molecular Devices Co., Tokyo, Japan) and the half-maximal inhibitory concentration of viability (IC50) was derived.

TiO_2 transferred into cellular components

Five ml of cell suspension of CHO cells and RBL-2H3 cells (5×10^4 cells/ml) or NHSF cells (4×10^4 cells/ml) was cultured in a 60-mm dish for 2 days under 5% CO_2 at 37°C. After removing the medium, 5 ml of 0.1 or 0.5 or 2 mg/ml TiO_2 suspened in culture medium was added and the mixture was incubated for a further 24 hr. After washing cells 5 times with medium, the cells were treated with trypsin and collected. The cells were suspended in 1.5 ml of phosphate-buffered saline (PBS) and ultrasonicated for 5 min. Then, cell suspensions were cell-fractionated in accordance with the scheme shown in Fig. 1 and the

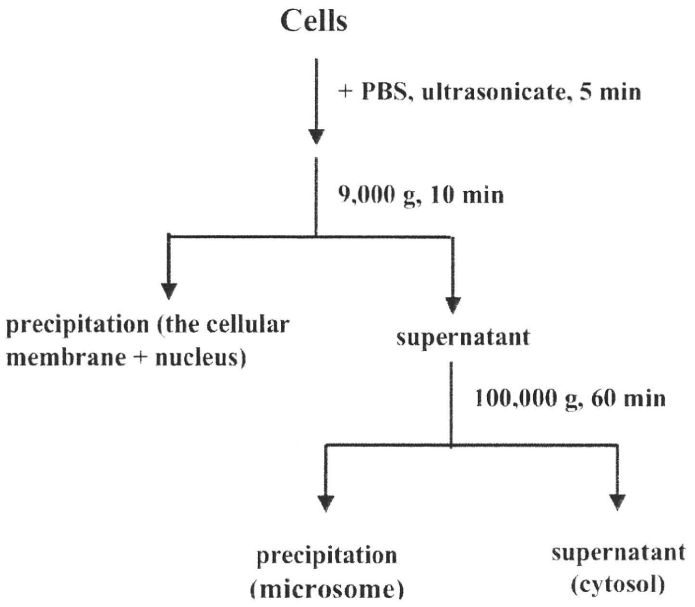

Fig. 1. Method of cell fractionation.

levels of Ti in the cellular membrane (including nucleus), microsome, and cytosol were determined by ICP-MS.

Statistical analysis

Differences between A431 and NHSF, CHO, or RBL-2H3 group, non-coated TiO_2 treatment group, and surface-treated TiO_2 treatment group were examined by Student's t-test. The differences were considered significant at $P < 0.05$.

RESULTS

Cytotoxicity of TiO_2 against cultured cells

LU175 showed cytotoxicity against NHSF, CHO, and RBL-2H3. On the other hand, LU175 showed no cytotoxicity against A431, B16 melanoma, and NHEK(F). IC50s of LU175 against NHSF, CHO, and RBL-2H3 were significantly decreased from that of the A431 group. LU205 showed no cytotoxicty against any cells (Table 1). IC50s of surface-treated TiO_2 were significantly higher than those of non-treated TiO_2 against NHSF and RBL-2H3 cells (Table 2).

Transfer of TiO_2 into the cellular components

500-10,000 μg of LU175 or LU205 was added to a culture of CHO cells in a 60-mm dish. After 24 hr, over 99% of LU205 was located in the cellular membrane, while 88.5-96.7% and 3.3-11.3% of LU175 were located in cellular membrane and cytosol, respectively (Table 3). The proportion of LU175 located in the cytosol was significantly increased from that in the LU205 group (Table 3).

The suspensions of 100 μg/ml surface-treated and non-treated TiO_2 were added to the NHSF seeded in a 60-mm dish. In non-treated TiO_2, proportions of MT-500B and LU175 located in cytosol were significantly increased from that in the LU205 group (Table 4). In surface-treated TiO_2, proportions of TTO-55(C) and SMT-500SAS located in cytosol were significantly increased from that in the MT-500SA group (Table 4). Similar results were obtained for RBL-2H3 cells (Table 4).

DISCUSSION

Xu *et al.* (2010) reported that nano-scale TiO_2 was administered to wild-type rat by a novel intrapulmonary spraying five times over 9 days, 8-hydroxyguanosine level and superoxide dismutase activity expression in the lung. Gurr *et al.* (2005) and Onuma *et al.* (2009) reported that reactive oxygen generated from TiO_2 in the dark induced cytotoxicity. Thus, the mechanism of the cytotoxicity of LU175 might be through reactive oxygen generated from TiO_2 in the dark. However, it was not clear that the kind of reactive oxygen generated from TiO_2 in the dark. Therefore, further study should be carried out.

Table 1. Cytotoxicity of LU175 and LU205 against various cells

Cell	IC50 (mg/ml)	
	LU175	LU205
CHO	1.3 ± 0.3*	> 10
RBL-2H3	1.4 ± 1.0*	8.6 ± 1.2
NHSF	0.5 ± 0.2**	> 10
A431	8.3 ± 2.9	7.0 ± 2.6
NHEK (F)	> 10	> 10
B16 melanoma	> 10	> 10

Data are means ± S.D. of three experiments. Significantly different from A431 group, *P < 0.05, **P < 0.01.

Table 2. Cytotoxicity of TiO_2 against NHSF and RBL-2H3 cells

	IC50 (mg/ml)	
	NHSF	RBL-2H3
SMT-500SAS	4.6 ± 0.4***	4.9 ± 1.9*
MT-500SA	> 10	> 10
MT-500H	> 10	5.9 ± 1.4**
TTO-55 (C)	2.1 ± 1.5	2.3 ± 1.4
TTO-55 (A)	> 10	> 10
MT-500B	0.12 ± 0.04	0.6 ± 0.4

Data are means ± S.D. of three experiments. Significantly different from MT-500B group, *P < 0.05, **P < 0.01, ***P < 0.001.

Table 3. The distribution of different particle sizes of TiO_2 in the cellular components of CHO cells

	Exposed (μg)	Detected (μg: mean ± S.D. n = 3)		
		Membrane	Microsome	Cytosol
LU175	500	83 ± 3	ND (< 0.004)	4.1 ± 2.7
		(95.3 ± 3.4%)	(0.0%)	(4.7 ± 3.1%)
	2500	176 ± 15	ND	6.0 ± 2.4*
		(96.7 ± 8.2%)	(0.0%)	(3.3 ± 1.3%*)
	10000	133 ± 17	0.28 ± 0.12	17 ± 14
		(88.5 ± 11.3%)	(0.2 ± 0.08%)	(11.3 ± 9.3%)
LU205	500	416 ± 8	2.4 ± 0.7	0.050 ± 0.001
		(99.41 ± 1.91%)	(0.57 ± 0.17%)	(0.02 ± 0.0004%)
	2500	1238 ± 25	3.6 ± 1.1	0.05 ± 0.08
		(99.71 ± 2.01%)	(0.29 ± 0.09%)	(0.004 ± 0.007%)
	10000	5395 ± 836	0.28 ± 0.11	0.61 ± 0.08
		(99.98 ± 15.49%)	(0.005 ± 0.002%)	(0.01 ± 0.001%)

The data detected after 24-hr exposure of TiO_2 onto CHO cells. Significantly different from LU205 group, *P < 0.05.

The cell size of NHSF, CHO, and RBL-2H3 cells were larger than B16 melanoma, A431 cells, and NHEK(F). Therefore, it was speculated that the cytotoxicity of LU175 might be connected to cell size. Surface-treated TiO_2 showed less cytotoxicity against RBL-2H3 cells and NHSF than non-treated TiO_2.

These results suggested that surface treatment with coating materials would reduce the surface reactive activity of TiO_2 and thus reduce its cytotoxicity. On the other hand, the cytotoxicity of surface-treated TiO_2 depends on the kind of coating material. The cytotoxicities of coating materials themselves are not known, so further study should be carried out.

Dose-dependent levels of LU175 were present in the cytosol of CHO cells. MT-500B and LU175 located in the cytosol of NHSF and RBL-2H3 cells were significantly increased from those in the LU205 group (Tables 3 and 4).

These results suggested that non-coating TiO_2 locat-

Table 4. Influence of surface coating on the distribution of TiO_2 in the cellular components of NHSF and RBL-2H3 cells

(a) NHSF (unit: μg)

TiO_2 500 μg	SMT-500SAS	MT-500SA	MT-500H	TTO-55(C)	TTO-55(A)	MT-500B	LU175	LU205
Cellular membrane	15.8 ± 1.4	117 ± 4	68.5 ± 3.8	3.55 ± 0.04	76.0 ± 2.3	83.2 ± 4.7	84 ± 5	426 ± 26
Microsome	0.26 ± 0.17	3.89 ± 0.26	0.92 ± 0.20	1.88 ± 0.06	ND	2.64 ± 0.24	23 ± 13	ND
Cytosol	1.92 ± 0.11	0.43 ± 0.04	2.85 ± 0.18	2.98 ± 0.18	0.59 ± 0.01	0.72 ± 0.07	2.8 ± 1.6	1.0 ± 0.7

(mean ± S.D., n = 3)

(presence ratio: %)

TiO_2 500 μg	SMT-500SAS	MT-500SA	MT-500H	TTO-55(C)	TTO-55(A)	MT-500B	LU175	LU205
Cellular membrane	87.88 ± 7.78	96.44 ± 3.30	94.78 ± 5.26	42.2 ± 0.5###	99.23 ± 3.00	96.11 ± 5.43	76.4 ± 4.6**	99.8 ± 6.1
Microsome	1.45 ± 0.95	3.21 ± 0.21	1.27 ± 0.28	22.4 ± 0.7	ND	3.05 ± 0.27	20.9 ± 11.8	ND
Cytosol	10.67 ± 0.61###	0.35 ± 0.03	3.95 ± 0.25	35.4 ± 2.1###	0.77 ± 0.01	0.84 ± 0.08**	2.5 ± 1.5	0.20 ± 0.16

(mean ± S.D., n = 3)

(b) RBL-2H3 (unit: μg)

TiO_2 500 μg	SMT-500SAS	MT-500SA	MT-500H	TTO-55(C)	TTO-55(A)	MT-500B	LU175	LU205
Cellular membrane	36.9 ± 18.7	283 ± 63	116 ± 48	32.0 ± 22.2	73.9 ± 5.8	89.3 ± 23.3	383 ± 39	471 ± 71
Microsome	0.24 ± 0.12	3.1 ± 1.5	1.3 ± 0.3	0.18 ± 0.27	0.91 ± 0.41	0.53 ± 0.17	3.1 ± 0.4	6.1 ± 0.6
Cytosol	0.63 ± 0.08	1.10 ± 0.06	3.61 ± 0.14	1.17 ± 0.09	0.86 ± 0.03	0.79 ± 0.06	0.54 ± 0.11	0.17 ± 0.13

(mean ± S.D., n = 3)

(presence ratio: %)

TiO_2 500 μg	SMT-500SAS	MT-500SA	MT-500H	TTO-55(C)	TTO-55(A)	MT-500B	LU175	LU205
Cellular membrane	97.7 ± 49.5	98.54 ± 21.94	95.94 ± 39.70	95.95 ± 66.57	97.66 ± 7.66	98.54 ± 25.71	99.05 ± 10.09	98.69 ± 14.88
Microsome	0.64 ± 0.32	1.08 ± 0.52	1.08 ± 0.25	0.54 ± 0.81	1.22 ± 0.54	0.58 ± 0.32	0.81 ± 0.10	1.27 ± 0.13
Cytosol	1.66 ± 0.21###	0.38 ± 0.02	2.98 ± 0.12	3.51 ± 0.27###	1.14 ± 0.04	0.87 ± 0.07***	0.14 ± 0.03*	0.04 ± 0.03

(mean ± S.D., n = 3)

Significantly different from LU205 group, *P < 0.05, **P < 0.01, ***P < 0.001.
Significantly different from MT-500SA group, ###P < 0.001

ed in the cytosol might be the main cause of cytotoxicity. Since the distribution in the cytosol of surface-treated TiO_2 is at the same level or higher than that of non-coating TiO_2, the distribution in the cytosol of surface-treated TiO_2 might not be the cause of the cytotoxicity of TiO_2. Since the distributions in the cytosol of TTO-55(C) and SMT-500SAS were significantly increased from that in the MT-500SA group, stearic acid or silicone induced the distribution in the cytosol.

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Original Article

Safety evaluation of titanium dioxide nanoparticles by their absorption and elimination profiles

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ABSTRACT — If titanium dioxide nanoparticles are inert and non-biodegradable, they must be evaluated similarly to fullerenes, carbon nanotubes and asbestos. We surveyed the titanium level in typical raw food materials, and then intravenously injected titanium dioxide nanoparticles (primary particle diameter: 15 nm; secondary particle size: 220 nm) in mice and determined their tissue distribution and elimination. As a result, an unexpectedly high titanium concentration was observed in several foods. It was also detected in blood and tissues of healthy mice without administration of titanium dioxide nanoparticles. Then, forced *i.v.* injection of the nanoparticles was performed in mice. The titanim level was significantly increased in blood and tissues, but no increase was found in the brain after *i.v.* injection. Most titanium was concentrated in the liver after injection, but the liver level decreased over time (ca. 30% decrease in 1 month). These data show that titanium must be eliminated from the body, and suggest that we should reconsider an evaluation method for toxicity of titanium dioxide nanoparticles.

Key words: Titanium dioxide, Nanoparticle, Absorption, Elimination

INTRODUCTION

Many nanomaterials have been prepared and evaluated for their functions and physical and chemical properties. In particular, biocompatible materials, ultrafine microstructures, and molecularly recognized and signaling materials have been broadly studied as leading-edge and advanced materials; fullerenes and carbon nanotubes are typical examples. In addition, titanium dioxide and zinc oxide nanoparticles are used not only in several materials but also in humans in UV-care cosmetics. These nanomaterials may be categorized as non-biodegradable, and liposomes and nano- and micro-emulsions are found in labile nanoparticles. Are these nanomaterials safe for humans (Maynard *et al.*, 2006; Behling, 2007; Scientific Committee on Consumer Products, 2007; Scientific Committee on Emerging and Newly-Identified Health Risks, 2007)?

Titanium and titanium dioxide are believed to be highly inert and safe, even when they are absorbed via the GI tract and skin. If they are inert and non-biodegradable, we should evaluate the titanium dioxide nanoparticles similarly to fullerenes, carbon nanotubes (Singh *et al.*, 2006) and asbestos. It was found from our preliminary survey and experiments, however, that titanium is naturally contained in the body as well as in vegetables and soil, indicating that we may ingest titanium compounds daily despite no detailed information on the chemical structure of such compounds. In addition, size is very important for nonbiodegradable nanoparticles. Titanium dioxide particles bigger than 100 nm have been used in several foods and toothpastes (Lomer *et al.*, 2005); thus our strategy to estimate the safety of titanium oxide nanoparticles must be modified.

In the present study, therefore, we surveyed the titanium level in typical raw food materials, and then intravenously injected titanium dioxide nanoparticles (primary particle size, ca. 15 nm; secondary particle size, ca. 220 nm: see below in detail) into mice, and determined the tissue distribution and elimination kinetics of titanium.

MATERIALS AND METHODS

Surveillance and determination method of titanium concentration in typical foodstuffs

Typical cooking ingredients were selected, and their titanium concentration was measured using an ICP-MS (Agilent 7500ce, Agilent Technologies, Inc., Santa Clara,

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