Tumorigenesis and Neoplastic Progression

Nano-Scaled Particles of Titanium Dioxide Convert Benign Mouse Fibrosarcoma Cells into Aggressive Tumor Cells

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Nanoparticles are prevalent in both commercial and medicinal products; however, the contribution of nanomaterials to carcinogenesis remains unclear. We therefore examined the effects of nano-sized titanium dioxide (TiO₂) on poorly tumorigenic and nonmetastatic QR-32 fibrosarcoma cells. We found that mice that were cotransplanted subcutaneously with QR-32 cells and nano-sized TiO₂, either uncoated (TiO₂-1, hydrophilic) or coated with stearic acid (TiO₂-2, hydrophobic), did not form tumors. However, QR-32 cells became tumorigenic after injection into sites previously implanted with TiO₂-1, but not TiO₂-2, and these developing tumors acquired metastatic phenotypes. No differences were observed either histologically or in inflammatory cytokine mRNA expression between TiO₂-1 and TiO₂-2 treatments. However, TiO₂-2, but not TiO₂-1, generated high levels of reactive oxygen species (ROS) in cell-free conditions. Although both TiO₂-1 and TiO₂-2 resulted in intracellular ROS formation, TiO₂-2 elicited a stronger response, resulting in cytotoxicity to the QR-32 cells.

Moreover, TiO₂-2, but not TiO₂-1, led to the development of nuclear interstices and multinucleate cells. Cells that survived the TiO₂ toxicity acquired a tumorigenic phenotype. TiO₂-induced ROS formation and its related cell injury were inhibited by the addition of antioxidant N-acetyl-L-cysteine. These results indicate that nano-sized TiO₂ has the potential to convert benign tumor cells into malignant ones through the generation of ROS in the target cells. (Am J Pathol 2009, 175:2171-2183; DOI: 10.2353/ajpath.2009.080900)

Nanomaterials are produced in tonnage quantities in the world every year, mainly as a result of the recent advances in nanotechnologies.¹ Nanoparticles are defined as ultra-fine particles of a diameter less than 100 nm,² and are basically fabricated from metal and ceramic oxides. Carbon, silica and titanium dioxide are the most frequently used materials to create nanoparticles due to their superior physical characteristics, particularly a very large surface-to-volume ratio,^{1,3} and several products made from these materials are already consumer goods. Application of nanomaterials has also been expanded to the medical fields; they are now recognized as promising new devices and applied in areas of biotechnology and biomedicine that aim at disease diagnosis, or used as drug delivery materials.⁴ These nanotechnological inno-

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Dr. Tetsuhiko Yoshimura passed away on October 7, 2007. This work was one of his last interests. We pay tribute to his devotion.

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Table 1. Characteristics of Titanium Dioxide Nanoparticles

Nanoparticles	Crystal	TiO ₂ contents	Surface treatment	Surface area (m ² /g)	Nominal size (nm)		Oil absorption (g/100 g)	pH	Hydrophathy
					Minor axis	Major axis			
TiO ₂ -1	rutile	84%–92%	ZrO ₂ Al(OH) ₃	70–90	40–70	200–300	40–55	6.5–8.0	Hydrophilic
TiO ₂ -2	rutile	77%–86%	ZrO ₂ Al(OH) ₃ and stearic acid	25–45	40–70	200–300	29–45	-	Hydrophobic

vations in medicine are referred to as “nanomedicines”⁵: nanoparticles are used for biomaterials (tissue or organ mimetic), interchangeable DNA parts (nanomachines), as well as for delivery of pharmaceutical, therapeutic, and diagnostic agents (nanotherapeutics).⁶

Among the manufactured nanoparticles, titanium dioxide (TiO₂) is the earliest industrially produced material.⁷ It was initially used as a photocatalyst,⁸ not only for air and water cleaning, but also for photodynamic tumor therapy.^{9,10} Nano-TiO₂ makes a good opacifier, and is used in many daily products, such as white pigment in paints, paper, plastics, food colorants, and cosmetics, especially sunscreens.^{7,11,12} Toxicity of coarse and micron-sized TiO₂ (larger than 100 nm in diameter) has been thought biologically inert in both human and animals^{13–16}; however, around 50% of all TiO₂-exposed workers have been reported to suffer from respiratory symptoms with impairment of pulmonary function.¹⁷

Nano-TiO₂-mediated toxicity has been increasingly reported, especially in relation to the induction of inflammation.¹⁸ It is known that toxicity increases with downscaling of particle sizes to small clusters of atoms.⁵ Indeed, nanoparticles are biologically more active and cause greater inflammatory reactions than larger particles.¹⁹ Therefore, finer sized particles may bring about unexpected and novel effects that are not observed in bulk or larger-scale particles.²⁰ However, current environmental laws and occupational health guidelines specify only the existing materials, and nano-sized particles evade regulation.¹

The objective of the present study was to determine the potential effects of nano-sized TiO₂ particles on the acceleration of the carcinogenic process by using regressive tumor cells (QR clones).²¹ The regressive phenotype of QR clones is mediated by host immunity in normal mice,^{22,23} and tumorigenic QR clones^{24,25} inhibit host anti-tumor immunity through the production of high levels of prostaglandin E₂ (PGE₂), which suppresses the induction of cytotoxic T lymphocytes.²² Therefore, the tumorigenic potential of QR clones in mice can be estimated from measuring PGE₂ secreted by cells converted from QR cells *in vitro*. We chose to use this model because we could monitor the conversion of a regressive phenotype to a tumorigenic/metastatic one in the presence of inflammation in mice.^{24–30}

In this study, we demonstrate that nano-TiO₂ alters the morphology of regressive QR-32 cells and enhances their PGE₂-mediated tumorigenic properties through formation of intracellular reactive oxygen species (ROS), and that the associated biological and immunological

changes differ according to the surface treatment of nano-TiO₂.

Materials and Methods

Nanomaterials and Chemicals

Nano-TiO₂ (rutile crystal phase) was a generous gift, aimed for scientific investigation, from Ishihara Sangyo Kaisha LTD (Osaka, Japan). We used two types of nano-TiO₂ with different surface modifications: TiO₂-1 treated with ZrO₂Al(OH)₃, which is hydrophilic; and TiO₂-2, treated with ZrO₂Al(OH)₃ and stearic acid, which is hydrophobic. Both of these forms were generated by the alkaline and acidic leaching method. The precise composition and the chemical properties of these nanoparticles are summarized in Table 1. As these nanoparticles are highly dispersed, we suspended them into 1.5% carboxymethylcellulose (217277, Wako Pure Chemical Industries, Osaka, Japan), and since nano-TiO₂ aggregates spontaneously in carboxymethylcellulose solution, the suspension was vortexed and sonicated before it was used for experiments. *N*-acetyl-L-cysteine (017-05131) and aminoguanidine (014-02542) were obtained from Wako Pure Chemical Industries.

Regressive Tumor Cells

BMT-11, a transplantable fibrosarcoma, was induced in a C57BL/6 mouse with 3-methylcholanthrene, and its tumorigenic clone, BMT-11 cl-9, was subsequently isolated by limiting dilution. BMT-11 cl-9 cells were exposed to quercetin *in vitro*, which gave rise to a number of random subclones (QR cells).²¹ These cell spontaneously regressed when injected into normal syngeneic mice and the regressive phenotype was due to DNA hypermethylation induced by quercetin.²¹ The phenotype was stable since the cells did not acquire tumorigenicity or metastatic ability spontaneously in culture for several years (data not shown). The QR clones are not revertant from tumorigenic to normal cells, since they grow lethally in immunodeficient mice and show tumor cell properties as they conserve *in vitro* anchorage-independent and infinite growth.^{22,23}

One of these regressive cell clones, QR-32, was used in this study. QR-32 cells and derived cell lines were maintained in Eagle's minimum essential medium (Nissui Pharm., Tokyo, Japan) supplemented with 8% fetal bovine serum (1370978, GIBCO), sodium pyruvate, non-

essential amino acids, and L-glutamine, at 37°C, in a humidified 5% CO₂/95% air mixture.

In Vitro Characterization of QR-32 Cells and Derived Cell Lines

For *in vitro* cell growth analysis, cells were seeded into a 6-well plate (1×10^5 cells per well; 3506, Corning, NY) and the medium was changed every other day. The cells were harvested and counted every day from day 1 to day 7 using the trypan blue exclusion test; doubling time was calculated from the logarithmic phase of the growth curve. For evaluation of plating efficiency, 1×10^3 cells suspended in medium were plated into 60-mm dishes (430166, Corning) in triplicate. The dishes were incubated for 7 days, colonies were then fixed in Carnoy's fixative, stained with 0.1% crystal violet and scored. For determination of growth in soft agar, 2×10^2 cells were suspended in 1 ml medium containing 0.3% agar (0710-500G, MS technosystems, Japan) and 2× volume of fetal bovine serum, and applied onto the pre-solidified 0.6% agar (1 ml) in 6-well plates. Triplicate plates were prepared for each cell line and after 3 weeks of incubation, colonies larger than 0.1 mm in diameter were scored.

Mice

Five-week-old female C57BL/6 mice were obtained from Nippon SLC (Hamamatsu, Japan). All of the mice were maintained in specific pathogen-free conditions, lit from 6:00 AM to 6:00 PM, at $23 \pm 2^\circ\text{C}$ and $45 \pm 15\%$ humidity, and fed with mouse diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and UV-irradiated water in the Institute for Animal Experimentation, Yamagata University Graduate School of Medicine. Diet and water were available *ad libitum* throughout the experiment.

Experimental Procedures

The experimental protocol was approved by the Committee of the Institute for Animal Experimentation, Yamagata University School of Medicine (#06-074).

The mice (at 6 weeks of age) were used after 1 week of acclimatization. They were divided randomly into four groups. Group 1: QR-32 cells (1×10^5 cells/0.1 ml) were subcutaneously injected into mice. Group 2: Either TiO₂-1 or TiO₂-2 (5 mg/0.1 ml) were mixed at an equal volume with QR-32 cells (1×10^5 cells/0.1 ml), then 0.2 ml of the mixture were injected into the mice subcutaneously. Groups 3 and 4: Either TiO₂-1 or TiO₂-2 (5 mg/0.1 ml) was injected alone into the mice subcutaneously, and 30 days (Group 3) and 70 days (Group 4) later, QR-32 cells (1×10^5 cells/0.1 ml) were injected into the nano-TiO₂ implantation sites. Tumor diameters were measured twice a week during the experiment.

Establishment of Culture Cell Lines from the in Vivo Growing Tumors and Estimation of Metastatic Properties

To assess whether the developing tumors had acquired metastatic properties, we removed the subcutaneously growing tumors aseptically, disaggregated them mechanically with scissors and used them for establishing individual cell lines. The detailed procedure has been described elsewhere.²⁴ Each tumor cell line was then injected intravenously (1×10^6 cells) into mice. On day 35, the mice were sacrificed with excess inhalation of ether and metastatic nodules on the surface of the lungs and other organs were counted macroscopically.

Immunohistochemistry

Nano-TiO₂-implanted subcutaneous tissues were excised at the times indicated and fixed with Bouin's solution overnight, immersed sequentially in 50%, 75%, and 99% ethanol every 24 hours to remove picric acid, dehydrated, embedded in paraffin, sectioned at 4 μm , and mounted on glass slides either for Azan staining or immunohistochemistry. For immunostaining, after deparaffinization and rehydration, the tissue sections were incubated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase. After rinsing, the sections were incubated with mouse 8-hydroxy-2'-deoxyguanosine (8-OHdG) monoclonal antibody (MOG-100 at a concentration of 10 $\mu\text{g/ml}$) or mouse anti-4-hydroxy-2-nonenal (HNE) monoclonal antibody (MHN-100 at a concentration of 25 $\mu\text{g/ml}$; Nikken Foods, Fukuroi, Japan), respectively, in a humidified chamber at 4°C overnight. Then, the sections were incubated for 10 minutes with MAX-PO complex, which is a conjugation of amino acid polymer with the Fab' portion of the secondary antibody and peroxidase (414321, Histofine mouse stain kit; Nichirei, Tokyo, Japan). After a final rinse, specific immunolabeling was examined with the use of 3,3'-diaminobenzidine (415171, Nichirei, Japan), as the chromogen, which was placed on the tissues for a few minutes. Development of 3,3'-diaminobenzidine was stopped by washing the tissues in distilled water. The sections were dehydrated, mounted, and photographed.

Isolation of Natural Killer Cells and Cytotoxicity Assay

The detailed procedure for isolating natural killer (NK) cells from mouse spleen and the NK cell-mediated cytotoxicity assay were described previously.²² Briefly, the isolated NK cells were used as effector cells, and each tumor cell line was labeled with a fluorescent dye, PKH67, at a final concentration of 3.5 $\mu\text{mol/L}$ (PKH67GL-1KT, Sigma, Tokyo, Japan). After 3 washes, the labeled tumor cells were suspended in RPMI medium and incubated with the NK cells at an effector-to-target cell ratio of 200:1 in a 96-well round-bottomed plate (3360, Corning) for 6 hours. The plate was centrifuged and the fluores-

cence intensity in the supernatants was measured at an excitation wavelength of 551 nm and an emission wavelength of 567 nm in a SpectraMax Gemini EM (01436, Molecular Devices, Tokyo, Japan). Spontaneous release and maximum release were determined by incubating target tumor cells without effectors in medium alone or in 1N hydrochloric acid, respectively. The specific percentage cytotoxicity was calculated as described below:

Specific NK cytotoxicity (%) = (fluorescence intensity release due to the cytotoxicity of the NK cells – value of spontaneous release from target cells)/(fluorescence intensity caused by treatment with 1N hydrochloric acid – value of spontaneous release from target cells) $\times$ 100.

Measurement of Mouse Vascular Endothelial Growth Factor, Transforming Growth Factor- β , and PGE₂ in Conditioned Medium by Enzyme-Linked Immunosorbent Assay

Commercially available enzyme-linked immunosorbent assay kits for mouse vascular endothelial growth factor (MMV00, R & D Systems, Minneapolis, MN), mouse transforming growth factor (TGF)- β (MB100, R & D Systems), and PGE₂ (EA 02, Oxford Biomedical Research, Metamora, MI) were used to quantify the level of each mediator in conditioned media according to the manufacturer's instructions. For PGE₂ samples, 1×10^5 cells were cultured in 24-well plates (3526, Corning) in 2 ml medium. After 24 hours, the supernatants were obtained. For TGF- β and vascular endothelial growth factor samples, 4×10^5 cells were cultured in 24-well plates in 0.5 ml medium for 24 hours. TGF- β samples were acid-activated and neutralized, and the resultant immunoreactive forms were generated just before enzyme-linked immunosorbent assay analysis.

RNA Extraction and cDNA Preparation

Frozen tissues were crushed to a powder in a mortar with liquid nitrogen. Total RNA was extracted using TRIzol (15596-018, Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. For real-time PCR, 1 μ g of total RNA was subjected to cDNA synthesis in 10 μ l of reaction mixture containing PrimeScript buffer consisting of dNTP mixture, MgCl₂, 25 pM/L oligo dT primer and 50 pM/L random 6-mers, and PrimeScript reverse transcription enzyme mix I (RR037A, PrimeScript RT reagent kit, Takara, Otsu, Japan). The reverse transcription reaction was performed sequentially for 15 minutes at 37°C, for 5 seconds at 85°C, and thereafter at 4°C.

Primer Design

Oligonucleotide primers were designed with the use of Primer 3 software version 0.4.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The primers were designed to produce an approximately 150-bp amplicon using the cDNA of interleukin (*IL*)-1 β (P10749), *IL*-2 (P04351), *IL*-4 (P07750), *IL*-6 (Q0PMN1), *IL*-10 (Q0VBJ1),

interferon (*IFN*)- γ (P01580), *TGF*- β (P04202), and *tumor necrosis factor*- α (Q3U593). To avoid amplification of genomic DNA, primers were placed within different exons close to an intron-exon boundary, with the probe spanning two neighboring exons whenever possible. The *β -actin* (Q6IWE2) gene was used as an endogenous control to normalize for differences in the amount of total RNA present in each sample. Primer sequences for the amplification of above genes by real-time PCR were as follows:

IL-1 β , sense, 5'-CCTCACAAGCAGAGCACAAg-3'; antisense, 5'-TGGGGAAGGCATTAGAAACA-3'; *IL*-2, sense, 5'-CCCACTTCAAGCTCCACTTC-3'; antisense, 5'-GGAGCTCTGTAGGTCCATC-3'; *IL*-4, sense, 5'-TCAACCCC-CAGCTAGTTGTC-3'; antisense, 5'-TGTGACCTCGTTC-AAAATGC-3'; *IL*-6, sense, 5'-AAGCGAGAGTCCTTCAGAGAGA-3'; antisense, 5'-GAGCATTGGAAATTGGGGTA-3'; *IL*-10, sense, 5'-CTGTTTCCATTGGGGACACT-3'; antisense, 5'-AAGTGTGGCCAGCCTTAGAA-3'; *IFN*- γ , sense, 5'-GAGGAACTGGCAAAAGGATG-3'; antisense, 5'-GCTGATGGCCTGATTGTCTT-3'; *TGF*- β 1, sense, 5'-ATTCTGGCGTTACCTTGG-3'; antisense, 5'-AGCCCTGTATCCGTCTCCT-3'; *Tumor necrosis factor*- α , sense, 5'-ACGGCATGGATCTCAAAGAC-3'; antisense, 5'-AGATAGCAAATCGGCTGACG-3'; and, *β -actin*, sense, 5'-TGAGGAGCACCTGTGCT-3'; antisense, 5'-ACATGGCTGGGGTGTGAAG-3'.

Quantitative Real-Time PCR

Real-time PCR experiments were performed using a commercial kit (QPK-201, SYBR Green Realtime PCR master mix, Toyobo, Osaka, Japan). Each reaction tube contained template DNA (200 ng) and 0.4 μ mol/L each of forward and reverse primers. A negative control was assembled by using the same concentrations of reagents but omitting the template DNA (data not shown). Samples were amplified in a thermocycler (LightCycler 2.0 Instrument, Roche Applied Science, Tokyo, Japan) for 40 cycles: 1 minute at 95°C, 5 seconds at 60°C, and 10 seconds at 72°C. Comparative Ct (Fit Points method) was used to calculate the expression level of individual genes using LightCycler, Software (Ver. 3.5, Roche Diagnostics, Tokyo, Japan).

Reverse Transcription-PCR Analysis

A detailed description of reverse transcription (RT)-PCR for thymosin β 4 or glyceraldehyde-3-phosphate dehydrogenase gene amplification has been described previously.²³

Detection of Cell-Free Reactive Oxygen Species

The generation of reactive oxygen species in cell-free conditions was determined by using the 2', 7'-dichlorodihydrofluorescein diacetate reagent (D-399, Molecular Probes, Tokyo, Japan). Nanoparticles were added to serum-free Eagle's minimum essential medium containing 10 μ mol/L dichlorodihydrofluorescein, which was

converted from H₂DCFDA by treatment with 0.1 M/L NaOH. An oxidized fluorescent product, dichlorofluorescein, was detected on a fluorescence reader, SpectraMax at an excitation of 488 nm and an emission of 530 nm, and monitored every 5 minutes. To confirm ROS production in the experimental system, 10 mmol/L of the antioxidant *N*-acetyl-L-cysteine was added.

Detection of Intracellular Reactive Oxygen Species Formation

The formation of intracellular ROS was measured as described above. Five $\times 10^3$ QR-32 cells were plated on 96-well plates (3340, Corning) and allowed to attach overnight. The cells were then washed twice with PBS and incubated with nanoparticles (312 μ g/ml) and 25 μ mol/L cell-permeable 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (C-6827, Molecular Probes) for 40 minutes. After the cells were washed with PBS, the plates were further incubated for 1 hour at 37°C, in a humidified 5% CO₂/95% air mixture incubator; the fluorescence intensities were measured at a 504 nm-excitation and a 530 nm-emission in the fluorescence reader.

Detection of Nitric Oxide in the Culture Supernatants

Since total nitrosothiols are decomposed into NO⁺ or NO, and the former further into nitrite (NO₂⁻), the production of nitric oxide (NO) was determined by measuring nitrite (NO₂⁻) and nitrate (NO₃⁻) concentrations in the culture medium using an NO detector high performance liquid chromatography column (ENO10; EICOM Corp., Kyoto, Japan) combined with a flow-reactor system. In this system, an in-line copper-coated cadmium reduction column (NO-RED, EICOM) is also equipped to convert nitrate to nitrite, which can be detected using Griess reagent in a flow reactor. The precise methods have been reported previously.³¹

Two $\times 10^5$ QR-32 cells were plated on 24-well plates and incubated overnight. The attached cells were then treated with nano-TiO₂ for 48 hours. After treatment, supernatants were harvested and centrifuged at 14,000 rpm for 5 minutes, and stored at -80°C until use. Detailed methods for isolating peritoneal exudate cells from mice were described in a previous report.²⁹

Cytotoxicity of Nanoparticles in QR-32 Cells, and Tumorigenicity and Metastatic Ability of the Survived Cells

1 $\times 10^5$ QR-32 cells were plated in 24-well plates and further incubated overnight. The attached cells were then treated with nanoparticles for different time periods (from 1 hour to 48 hours). Suspensions of QR-32 cells with nano-TiO₂ (312 μ g/ml) were incubated for 72 hours. After the nano-TiO₂ exposure, cells were harvested using trypsin, and counted by the trypan blue exclusion method.

The cells that survived after 48 hours of treatment were cultured further and an aliquot of these cells was expanded for subcutaneous (2×10^5 cells) or intravenous (1×10^6 cells) injection into the mice. Another aliquot of the treated cells was repeatedly treated with nano-TiO₂ for 48 hours with or without *N*-acetyl-L-cysteine (NAC) treatment (195 μ mol/L).

Transmission Electron Microscopy

The QR-32 cells were co-cultured with nano-TiO₂ (312 μ g/ml) overnight, collected with a rubber policeman and centrifuged at 1200 rpm for 5 minutes. After washing twice in 2% sucrose buffered with 0.05 M/L sodium cacodylate (pH 7.4), they were fixed with 2.5% glutaraldehyde in the same buffer for 15 minutes at 4°C. The cells were then washed with the same buffer and postfixed for 10 minutes with a 1% osmium tetroxide in the same buffer solution. Subsequently they were dehydrated in graded series of ethanol every 10 minutes, immersed twice in propylene oxide for 10 minutes, and embedded in epoxy resin. Ultra thin sections on copper grids were stained with uranyl acetate and lead citrate, and then photographed by a transmission electron microscope (H-7100, Hitachi high-technologies, Japan) equipped with a digital charge-coupled device camera (Olympus soft image solutions GMBH, MegaView III, Germany).

Statistical Analysis

The significance of the differences in tumor and metastatic incidences was calculated by the χ^2 test and the differences in cytokine/growth factor expressions, cell numbers and NO₂⁻/NO₃⁻ concentration were evaluated using the Student's *t*-test.

Results

Regressive to Tumorigenic Conversion of QR-32 Cells Following Injection in Titanium Dioxide Nanoparticle-Implanted Sites

To examine whether titanium dioxide nanoparticles (nano-TiO₂) have the potential to accelerate carcinogenesis and tumor progression, we used two types of nano-TiO₂, TiO₂-1, and TiO₂-2; the former was made hydrophilic, and the latter hydrophobic, by surface treatments (Table 1).

Table 2 shows stimulating effects of nano-TiO₂ on tumor formation. A clonal mouse fibrosarcoma, QR-32 cells, exhibited spontaneous regression after subcutaneous injection (1×10^5 cells) into normal syngeneic C57BL/6 mice. None of the mice developed tumors following simultaneous co-implantation of QR-32 cells with TiO₂-1 or TiO₂-2; however, QR-32 cells injected into a subcutaneous site where TiO₂-1 had been implanted for 30 or 70 days grew lethally in 5 out of 15 mice (33%) and 8 out of 15 mice (53%), respectively. Interestingly, tumorigenicity was significantly increased when QR-32 cells

Table 2. Tumorigenicity of QR-32 Cells Injected into Nanoparticle-Implanted Sites

Days after nanoparticle implantation*	QR-32 cells (1×10^5) implanted into [†]	Incidence of tumorigenicity (Number of tumors/tested mice)			Mean days before tumor diameter reached 10 mm
		Exp. I	Exp. II	Total	
NA ^{‡§} 0 (simultaneous)	QR-32 alone	0/5	0/10	0/15	NA
	Vehicle	0/5	0/10	0/15	NA
	TiO ₂ -1	0/5	0/10	0/15	NA
30	TiO ₂ -2	0/5	0/10	0/15	NA
	Vehicle	0/5	0/10	0/15	NA
	TiO ₂ -1	2/5	3/10	5/15 [¶]	60.2 ± 21.9
70	TiO ₂ -2	0/5	1/10	1/15	50
	Vehicle	0/5	0/10	0/15	NA
	TiO ₂ -1	3/5	5/10	8/15	40.1 ± 7.9
	TiO ₂ -2	0/5	1/10	1/15	27

*QR-32 cells were injected subcutaneously in the sites where the nanoparticles had been implanted for the indicated periods of time.

[†]QR-32 cells (1×10^5 cells/0.1 ml) in a PBS suspension were injected into the nanoparticles that had previously been implanted subcutaneously. The mice were observed for 3 months.

[‡]QR-32 cells (1×10^5 cells/0.1 ml) in a PBS suspension were injected into mice that had no nanoparticle implantation.

[§]NA, not applicable.

[¶] $P < 0.05$, ^{||} $P < 0.005$, vs. vehicle-injected group.

were injected into sites pre-implanted with TiO₂-1, but not TiO₂-2. At the time of sacrifice, there was no evidence of typical spontaneous metastasis in the mice. Moreover, there was no autologous tumor development in mice after subcutaneous injection of either TiO₂-1 or TiO₂-2 for at least 1.5 years, under our experimental conditions (data not shown).

Acquisition of Metastatic Ability in the Arising Tumors

It is the advantage of our model that we can determine whether the arising tumors acquire metastatic ability without further involvement of a potentially tumorigenic substance, since the tumor cell lines had been earlier established by culturing the cells from tumors derived from individual mice and so their metastatic potential can be examined in other mice. From the QR-32 cells injected into pre-implanted TiO₂-1 and TiO₂-2, we established 7 cell lines, QRnP-1~7, and 1 cell line, QRnP-8, respectively. All of the QRnP tumor cell lines significantly metas-

tasized to lungs as compared with QR-32 cells (Table 3). QRnP tumor cells also acquired extrapulmonary metastases such as ovary, greater omentum, and inguinal or subcutaneous lymph nodes. Some of the mice also exhibited pleuritis carcinomatosa.

Thymosin β 4 Gene Expression in QRnP Tumor Cells

We have previously determined that *thymosin β 4* gene expression was responsible for the acquisition of a metastatic phenotype in QR-32 cells.²³ We therefore analyzed whether the arising QRnP tumor cells express *thymosin β 4* gene. The parental QR-32 cells did not express *thymosin β 4*; however, 4 out of 8 QRnP tumor cell lines exhibited enhanced *thymosin β 4* expression at various levels (Figure 1A). We suspect that the metastatic cells without *thymosin β 4* expression may have had other gene alteration(s). QRsP-30 tumor cells that had progressed from QR-32 cells in the presence of inflammation showed *thymosin β 4* expression; they were used as a positive control.²³

Table 3. Acquisition of Metastatic Ability in the Arising Tumors

Cell line established from arising tumors	Number of mice with metastasis/no. of mice tested*		
	Lung colonizing ability		Other metastasis sites Sites (incidence)
	Incidence	Number of lungs with metastatic nodules	
QR-32	0/5	0, 0, 0, 0, 0	None
QRnP-1	5/5 [†]	4, 4, 13, 26, 41	Ovary (2/5); pleuritis carcinomatosa (2/5)
QRnP-2	5/5 [†]	3, 3, 5, 8, 12	Ovary (1/5); greater omentum (1/5)
QRnP-3	5/5 [†]	11, 15, 20, 20, 27	Ovary (1/5); inguinal lymph node (1/5)
QRnP-4	4/6 [‡]	0, 0, 4, 12, 19, >150	Ovary (3/6)
QRnP-5	3/5 [‡]	0, 0, 2, 3, 16	Ovary (1/5)
QRnP-6	3/5 [‡]	0, 0, 8, 17, 32	Ovary (1/5); pleuritis carcinomatosa (2/5)
QRnP-7	5/5 [†]	4, 45, 46, 49, 85	Ovary (3/5 [‡]); greater omentum (1/5)
QRnP-8	4/5 [‡]	0, 10, 13, 23, 40	Ovary (1/5); subcutaneous lymph node (1/5)

*One $\times 10^6$ cells from each cell line established from the arising tumors were injected intravenously into mice. Thirty-five days later, the mice were sacrificed and metastatic nodules at the surface of the lung were counted macroscopically.

[†] $P < 0.005$, [‡] $P < 0.05$, vs. QR-32 group.

Figure 1. Thymosin $\beta 4$ gene expression in QRnP tumor cells and detection of 8-OHdG and HNE in titanium dioxide nanoparticle implantation sites. **A:** Tumor cell lines were established from tumors derived from QR-32 cells that had been injected into the pre-implantation site of either TiO₂-1 (QRnP-1 to 7) or TiO₂-2 (QRnP-8) implanted mice. The Figure shows RT-PCR analysis for *thymosin β4* and *GAPDH* gene expression. **B:** Five mg of TiO₂-1 or TiO₂-2 were implanted subcutaneously in mice. Large (black arrowhead) and small (white arrowheads) deposits of the nanoparticles are indicated. Tissues were removed on the indicated days and stained by the Azan method or subjected to immunohistochemical study for detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 4-hydroxy-2-nonenal (HNE). Scale bar: 200 μ m.

Detection of 8-OHdG and HNE in Titanium Dioxide Nanoparticle Implantation Sites

Because nano-TiO₂ is recognized as foreign body in the host, we had initially considered the induction of inflammation in the host at the implanted site. Macroscopically, implanted nanoparticles were observed as packed white pigment at the implantation site for at least 70 days. Histologically, the particles were recognized as large deposits (filled triangle) or small clusters (triangle) (Figure 1B). The aggregation state of nano-TiO₂ showed a slight difference: TiO₂-1 tended to be closely packed, whereas TiO₂-2 was rather loosely aggregated and diffused. Also, fibrous stroma proliferated more in the TiO₂-1 implantation site than in the TiO₂-2 site, which we observed by Azan staining of collagen fiber formation around the TiO₂-1 implantation site. We next examined 8-OHdG adducts formed by reactive oxygen species (ROS) as markers of inflammatory reactions at the subcutaneous site where the mice had been implanted with nano-TiO₂ for 30 and 70 days. Relatively intense staining for 8-OHdG was observed in TiO₂-2-implanted sites compared with that in the TiO₂-1 sites. The intensity pattern of aldehyde HNE-modified proteins, which are known to be formed during lipid peroxidation, was similar to that of 8-OHdG staining. Both 8-OHdG- and HNE-positive cells were mostly stromal fibroblasts and infiltrated macrophages. The specificity of the antibody to 8-OHdG or HNE has been proved by incubating with 8-OHdG polynucleotide or normal mouse serum instead of HNE antibody, which results in disappearance of the positive staining.²⁶

Quantitative Real-Time PCR for Inflammation-related Cytokines and Growth Factors

We next quantified mRNA of inflammation-related cytokines and growth factors at the subcutaneous tissues with nano-TiO₂ implantation for 30 days and 70 days (Figure 2). TGF- β was up-regulated in TiO₂-1-implanted

tissues at Day 30, which probably induced collagen fiber (Figure 1B). We observed obvious differences in other cytokine and growth factor expressions; however, at present, we have not been able to discern any specific expression that closely correlated with the incidence of tumor development from QR-32 cells injected into pre-implanted TiO₂-1 or TiO₂-2.

Formation of Reactive Oxygen Species by Titanium Dioxide Nanoparticles in a Cell-Free System and in QR-32 Cells

The ability of nano-TiO₂ to generate ROS was assessed using fluorescein reagents. Generation of ROS by the nanoparticle itself (cell-free system) is shown in Figure 3A. TiO₂-1 did not generate ROS, whereas TiO₂-2 generated ROS in dose-dependent manner, and the increase of ROS was inhibited by the presence of the antioxidant N-acetyl-L-cysteine (NAC). Figure 3B demonstrates the

Figure 2. Quantitative real-time PCR for inflammation-related cytokines and growth factors. Real-time RT-PCR analysis was performed to quantify changes in mRNA expression of eight cytokine/growth factors in the TiO₂-1- or TiO₂-2-implanted tissues. *P < 0.05 and **P < 0.01 vs. TiO₂-1.

Figure 3. Formation of reactive oxygen species and mediation of cytotoxicity to QR-32 cells by titanium dioxide nanoparticles. **A:** Generation of reactive oxygen species (ROS) by nano-TiO₂ in a cell-free system was determined using the 2', 7'-dichlorodihydrofluorescein diacetate reagent. **P* < 0.001 vs. individual nano-TiO₂ treatment. ***P* < 0.05 and ****P* < 0.001 vs. medium alone. **B:** Intracellular ROS generation in QR-32 cells co-cultured with 312 μg/ml of nano-TiO₂ was determined using cell-permeable 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester. **P* < 0.05 and ***P* < 0.001 vs. QR-32 cells. **C:** Production of nitric oxide (NO) was determined by measuring both nitrite (NO₂⁻) and nitrate (NO₃⁻) concentrations in the culture medium. **P* < 0.05 and ***P* < 0.01 vs. QR-32 cell alone. Peritoneal exudate cells (PEC) were collected from murine peritoneal cavities 5 days after implantation of a piece of gelatin sponge and used as positive controls for NO production. **D:** Treatment of adherent (from 1 hour to 48 hours) or suspended (for 72 hours; 72S) QR-32 cells with nano-TiO₂ (312 μg/ml). After exposure to the nano-TiO₂, the cells were harvested and counted. **P* < 0.05 and ***P* < 0.001 vs. QR-32 cells. Closed bars: TiO₂-1 alone (**A**) or QR-32 cells treated with TiO₂-1 (**B**, **C**, **D**); open bars: TiO₂-2 alone (**A**) or QR-32 cells treated with TiO₂-2 (**B**, **C**, **D**); dotted bars: culture medium alone (**A**) or non-treated QR-32 cells (**B**, **C**, **D**). *N*-acetyl-L-cysteine (NAC) and aminoguanidine (AG) were used as inhibitors of ROS and NO, respectively.

capability of nano-TiO₂ to form intracellular ROS in QR-32 cells. QR-32 cells were naturally oxidized during cell culture, whereas ROS formation was significantly increased when the cells were co-cultured with nano-TiO₂ (312 μg/ml). TiO₂-2 generated more abundant ROS in QR-32 cells than TiO₂-1. This augmented ROS production was inhibited in the presence of NAC. Therefore, it was evident that nano-TiO₂ actually generated ROS within the QR-32 cells. We also confirmed that freshly diluted hydrogen peroxide induced ROS in the QR-32 cells in a dose-dependent manner.

Nitric Oxide Formation in the Supernatants of QR-32 Cells Co-Cultured with Nano-TiO₂

It has been reported that nano-TiO₂ produces not only ROS, but also NO in the cells.³² Moreover, once ROS are formed in the cells, they enhance cytosolic calcium concentration³³ and/or activate transcription factors, triggering the up-regulation of several pro-inflammatory genes, including the gene for inducible nitric oxide synthase.³⁴ To examine whether nano-TiO₂ forms NO, we measured nitrite (NO₂⁻) and nitrate (NO₃⁻) secreted into the medium of QR-32 cells co-cultured with nano-TiO₂, using high performance liquid chromatography coupled with flow reactor systems.³¹

QR-32 cells alone had a significant production of NO, compared with the medium alone, which was converted to NO₂⁻ and NO₃⁻ (Figure 3C). However, none of the nano-TiO₂ treatments significantly enhanced nitrite/nitrate concentration in the medium. As a positive control for this NO detec-

tion system, we measured peritoneal exudate cells (PEC) collected from the mice that had a foreign body implanted for 5 days.²⁹ While increased production of nitrite/nitrate was observed, this was inhibited by an inhibitor of inducible nitric oxide synthase, aminoguanidine (AG).

Acquisition of Tumorigenicity Mediated by Nano-TiO₂ in QR-32 Cells

We next examined whether nanoparticle-induced ROS cause cytotoxicity to QR-32 cells (Figure 3D). QR-32 cells were incubated on culture plates overnight, and the attached cells were treated with 312 μg/ml nano-TiO₂ for various durations, up to 2 days. We also examined cytotoxicity by simultaneously cultivating QR-32 cells with nano-TiO₂ for 3 days (simultaneous co-culture; 72S). TiO₂-2 had strong cytotoxic activity on attached QR-32 cells through ROS production because the cytotoxic activity was inhibited by the presence of NAC. Meanwhile, TiO₂-1-induced cytotoxic activity was weaker than that of TiO₂-2, and the number of the resultant proliferative cells after nano-TiO₂ treatment was much larger in TiO₂-1. Moreover, simultaneous co-culture of QR-32 cells with nano-TiO₂ showed the highest cytotoxic activity. This is one of the reasons why no tumors grew after simultaneous implantation (injection of a mixture of QR-32 cells and nano-TiO₂; Table 2).

We further examined whether the survived QR-32 cells, after co-cultivation with nano-TiO₂, acquire a malignant phenotype (Table 4). Significant tumorigenic

Table 4. Acquisition of Tumorigenicity of Survived QR-32 Cells after Titanium Dioxide Nanoparticle Treatment

QR-32 cell treatment	Number of mice with tumorigenicity/ no. of mice tested* Number of passages in culture			Number of mice with metastasis/ no. of mice tested† Number of passages in culture		
	1	2	3	1	2	3
Untreated	0/5	0/5	0/5	0/5	0/5	0/5
TiO ₂ -1	1/5	1/5	3/5 [‡]	1/5	0/5	0/5
TiO ₂ -1 + NAC	0/5	0/5	0/5	0/5	0/5	0/5
TiO ₂ -2	0/5	2/5	3/5 [‡]	0/5	0/5	0/5
TiO ₂ -2 + NAC	0/5	0/5	0/5	0/5	0/5	0/5

*After treatment with 312 µg/ml nano-TiO₂, the survived cells were grown in maintaining medium and an aliquot of the cells were expanded for subcutaneous injection (2 × 10⁵ cells) into mice.

†One × 10⁶ cells of the same aliquot of cells were injected intravenously into mice (2 × 10⁵ cells). Thirty-five days later, the mice were sacrificed and metastatic nodules at the surface of the lung were counted macroscopically.

[‡]P < 0.05, vs. non-treated QR-32 cells.

conversion (subcutaneous lethal growth) was observed after three passages of serial treatment with TiO₂-1 and TiO₂-2, respectively. The increase in tumorigenic incidences was inhibited by the presence of NAC. Meanwhile, acquisition of metastatic ability by nano-TiO₂ was not evident.

Effects of Nano-TiO₂ on the Biological Characteristics of Parental QR-32 Cells and Its Derived Cell Lines

Table 5 shows a comparison of biological features of the cells. All of the cell lines except parental QR-32 cells acquired subcutaneous tumorigenicity, and two of the cell lines (QR/TiO₂-1 or QR/TiO₂-2) failed to acquire metastatic ability. Intriguingly, tumors that arose from the primary tumor after subcutaneous injection of QR/TiO₂-1

or QR/TiO₂-2 cells acquired a metastatic phenotype (Table 5). PGE₂ is closely associated with lethal tumorigenicity of the cell lines. TGF-β is another immunosuppressive and/or angiogenic factor, which was increased in QR/TiO₂-1 and QRsP-30 cells but lost in QR/TiO₂-1-derived tumorigenic cell lines. Vascular endothelial growth factor production, *in vitro* characteristics and NK sensitivity of the cell lines showed some variations but were similar to those of parental QR-32 cells.

Transmission Electron Microscopic Examination

QR-32 cells cultured with nano-TiO₂ treatment were subjected to transmission electron microscopic examination. As shown in Figure 4, nano-TiO₂ formed clusters of electron-dense particles in the extracellular spaces. TiO₂-1

Table 5. Biological Characteristics of QR-32 Cells and Its Derived Cell Lines

Cell lines	<i>In vivo</i> Number of mice with tumor or metastasis/no. of mice tested		<i>In vitro</i>						
	Tumorigenicity*	Metastasis†	PGE ₂ production (ng/ml) [‡]	TGF-β production (ng/ml) [§]	VEGF production (pg/ml)	% cytotoxicity by NK cells	Doubling time (hours)**	Plating efficiency (%) ^{††}	Number of colonies in soft agar ^{††}
QR-32	0/5	0/5	2.6 ± 0.2	UD	819 ± 12	3.0 ± 0.5	23.0	37.5 ± 2.9	40.3
QR/TiO ₂ -1	3/5 ^{§§}	0/5	6.7 ± 0.2	116 ± 2	830 ± 17	5.0 ± 0.8 ^{§§}	27.0	38.6 ± 1.4	40.5
QR/TiO ₂ -1-P1	3/5 ^{§§}	5/5 ^{§§}	6.8 ± 0.1	UD	835 ± 31	3.4 ± 0.7	23.0	39.4 ± 0.6	35.7
QR/TiO ₂ -1-P2	3/5 ^{§§}	4/5	6.9 ± 0.1	UD	827 ± 14	2.9 ± 0.2	25.7	39.2 ± 2.5	50.5
QR/TiO ₂ -2	3/5 ^{§§}	0/5	6.7 ± 0.1	UD	797 ± 29	2.9 ± 0.2	23.0	39.2 ± 2.5	52.3
QR/TiO ₂ -2-P1	4/5	6/7	7.6 ± 0.2	UD	829 ± 25	2.2 ± 0.4	25.7	39.8 ± 0.7	43.2
QR/TiO ₂ -2-P2	3/5 ^{§§}	5/5	6.7 ± 0.3	UD	834 ± 18	2.7 ± 0.8	25.7	45.8 ± 0.6	37.0
QRnP-1	6/6	5/5	10.6 ± 0.5	UD	849 ± 26	1.8 ± 0.4 ^{§§}	24.3	42.2 ± 1.4	43.8
QRnP-2	6/6	5/5	8.4 ± 0.2	UD	816 ± 31	2.7 ± 0.2	23.0	39.4 ± 1.2	42.2
QRnP-3	5/5	5/5	8.5 ± 0.1	UD	832 ± 31	3.4 ± 1.2	23.9	42.4 ± 0.5 ^{§§}	41.3
QRnP-8	6/6	4/5	8.6 ± 0.2	UD	829 ± 22	4.3 ± 0.3 ^{§§}	21.2	41.3 ± 0.6	37.7
QRsP-30	5/5	5/5	10.8 ± 0.3	93 ± 3	904 ± 12	3.2 ± 0.2	25.3	39.7 ± 1.4	50.8

*Two × 10⁵ cells were injected subcutaneously into mice.

†One × 10⁶ cells were injected intravenously into mice. Mice were sacrificed 35 days later and metastatic nodules at the surface of lung were counted macroscopically.

[‡]One × 10⁵ cells were cultured in 24-well plates in 2 ml of medium. After 24 hours, the supernatants were collected and used for the PGE₂ assay.

[§]TGF-β levels in supernatants were measured from 4 × 10⁵ cells cultured in 24-well plates in 0.5 ml medium for 24 hours. The collected samples were acid-activated and neutralized just before ELISA analysis. UD, under detection limit (<31.2 pg/ml).

^{||}VEGF samples were collected and used in a manner same as that used for TGF-β samples without acid-activation.

^{||}Tumor cells were labeled with a fluorescent probe, PKH67, and co-cultured with NK cells obtained from syngeneic mice. After 6 hours, the fluorescent intensity of the supernatants was measured and specific cytotoxicity of tumor cells was calculated.

**One × 10⁵ cells were plated into 6-well plates and counted every day from day 1 to 7 by the trypan blue exclusion test.

††One × 10³ cells from each cell line were plated into 60-mm dishes and incubated for 10 days.

††Two × 10² cells were suspended in 1 ml 0.3% agar and plated on pre-solidified 0.6% agar in 6-well plates colonies formed in 0.6% agar were counted after 3 weeks in incubation.

^{§§}P < 0.05, ^{||}P < 0.01, vs. QR-32 cells.

Figure 4. Transmission electron microscopic examination of QR-32 cells after coculture with nano-TiO₂. Transmission electron microscopy revealed that hydrophilic nano-TiO₂ (TiO₂-1; **A, C, E**) was incorporated into enlarged follicle-like organelles in the cytoplasm of QR-32 cells. Hydrophobic nano-TiO₂ (TiO₂-2; **B, D, F**) formed interstices around the nuclear membrane and caused a multinucleated phenotype in QR-32 cells. Spontaneous aggregation of nano-TiO₂ was seen in the extracellular space. Original magnification: $\times 3000$ (**A**); $\times 3000$ (**B**); $\times 10,000$ (**C**); $\times 7000$ (**D**); $\times 15,000$ (**E**); and $\times 20,000$ (**F**). Scale bars = 5 μm (**A**); 5 μm (**B**); 2 μm (**C**); 5 μm (**D**); 1 μm (**E**); and 1 μm (**F**).

was incorporated into QR-32 cells and found in enlarged follicle-like organelles in the cytoplasm (Figure 4, A, C, and E). In contrast, TiO₂-2 formed interstices around the nuclear membrane (Figure 4, B, D, and F), and the TiO₂-2-treated QR-32 cells became multinucleated (Figure 4 B).

Discussion

In this study we reveal that pre-implanted nano-TiO₂ particles convert non-tumorigenic and nonmetastatic mouse fibrosarcoma cells into tumorigenic and metastatic ones. We found that the acquisition of malignant properties differed depending on the surface modification of nano-TiO₂ and that the conversion frequency may be explained by the degree of ROS formation in the cells.

The influence of material's hydrophathy has been pointed out in foreign-body-induced carcinogenesis, which may be fundamentally same in fine particle-related carcinogenesis.³⁵ In experimental foreign body-induced carcinogenesis, subcutaneous implantation of hydrophobic membranes in mice develops more autologous tumors than those obtained with hydrophilic ones.³⁶ Tumor development is said to depend on the host's immunological reaction and its

derived intermediate reactive substances to the foreign bodies.³⁵ We have revealed that ROS are commonly formed in QR-32 cells after co-culture with either hydrophilic or hydrophobic nano-TiO₂. ROS generation by TiO₂ is a well-known phenomenon³⁷ and the feature has been used for antibacterial activity against both Gram-negative and -positive bacteria.³⁸ It is noteworthy that the degree of intracellular ROS formation was apparently different between the two types of particles. In the present study, hydrophobic nano-TiO₂ formed a large quantity of ROS in the cells that caused cell death. On the other hand, the survived cell population that escaped from ROS-induced cytotoxicity was much larger in the hydrophilic particle-treated cells than in the hydrophobic particle-treated ones (Figure 3D). We have previously revealed that the survived cell population from benign QR-32 cells was converted into aggressive tumor cells by inflammatory cell-derived ROS- and NO-mediated injury.^{29,30} The QR-32 cells used in the study are a typically sensitive clone to ROS injury because they contain less antioxidative enzymes (low in manganese superoxide dismutase and glutathione peroxidase activities), compared with ROS-resistant clones.²⁸ Therefore, we assumed that ROS-affected surviving cells would be prone to convert themselves into more malignant ones.^{27,28,39}

There are at least six possible mechanisms responsible for ROS formation by nano-sized particles: i) Surface-catalyzing chemical reactions of the nanoparticles themselves⁴⁰; ii) Interfusion of redox-active transitional metals such as Fe, Co, Ni, and Cu into the particles forms ROS through a Fenton-type reaction⁴¹; iii) Interaction of nanoparticles with cell membranes stimulates ROS generation⁴²; iv) Ultra-fine particles can enter mitochondria and form ROS⁴³; v) Ultra-fine TiO₂ reduces glutathione levels in cells, and reduced antioxidative enzymes may conversely allow ROS formation⁴⁴; vi) UV irradiation stimulates ROS production by nano-TiO₂.⁴⁵ Moreover, it has been reported that not only ROS but also NO is produced by nano-TiO₂.³² However, in the current study involvement of UV irradiation or NO is negligible (Figure 3C). Immunohistochemical analysis revealed that hydrophobic nano-TiO₂ generated more oxidative by-products in tissues (Figure 1B). Besides, ROS formation by phagocytic immune effector cells may also be involved since hydrophobic nanoparticles are preferentially captured by tissue macrophages, which are then stimulated to form ROS secondarily.⁴⁶

We are aware that our experimental system is different from the actual usage conditions of nano-TiO₂, regarding both dosage and administration sites. Nevertheless, physiological barriers that protect the human body from foreign particles are reported to be against particles larger than 100 nm,^{47,48} and the ROS-mediated toxicity of the particles may depend on their ability to penetrate into tissues or cell organelles. Currently the depth of nano-TiO₂ penetration is still a contentious issue. When they are topically administered, the particles may be able to enter the stratum corneum⁴⁹ and cross cellular membranes through non-phagocytic mechanisms.⁵⁰ Moreover, incorporated nanoparticles may cross endothelial cells and migrate into the blood and lymph circulation.^{51,52} In the cell, the particles are not distributed uni-

formly in the cytoplasm; instead they rapidly gather within vacuoles,⁵³ and occasionally enter into nuclei.^{50,54} In this study, we demonstrate that there are apparent differences in the incorporation manner and/or morphology of QR-32 cells when they are treated with two types of nano-TiO₂ (Figure 4). Intriguingly, after addition of TiO₂-2, but not TiO₂-1, to QR-32 cells, cytomorphological alterations, such as diminution of microvilli, formation of interstices around the nuclear membrane, and a multinucleated phenotype, were evident (Figure 4B). These results coincided with nano-TiO₂-induced cytotoxicity. TiO₂-2 has high toxicity to QR-32 cells, presumably causing structural damages to the nuclei through production of a high amount of ROS. In contrast, TiO₂-1-treated QR-32 cells kept nearly normal morphology. A recent study revealed that a multinucleated phenotype is brought about by oncogenic signaling-dependent ROS production in melanocytes.⁵⁵ We have to determine whether ROS formation is due to activation of signaling cascade(s) besides ROS formation by the nanoparticles themselves.

There was a discrepancy between the *in vitro* and *in vivo* results; while *in vitro* TiO₂-1 and TiO₂-2 both generated tumorigenic variants (Table 4), *in vivo* only pre-implanted TiO₂-1 converted QR-32 cells into tumorigenic ones (Table 2). *In vitro*, the cells were exposed to the nanoparticles for 48 hours; then the nanoparticles were washed off, and the survived cells were allowed to grow until the next serial passages. Otherwise the cells would not have survived in the continuous presence of nanoparticles, especially TiO₂-2. It is conceivable that some of the cells adapted themselves to the condition and proliferated in culture, regardless of whether they had been treated with TiO₂-1 or TiO₂-2. Thus we observed the tumorigenic population in both TiO₂-1-treated and TiO₂-2-treated cells *in vitro*. In contrast to the *in vivo* condition, the cells were constantly affected by the nanoparticles, and survived cells were found only in the TiO₂-1-treated group, which, as we understand, indicates that TiO₂-2 is more cytotoxic than TiO₂-1. Thus the *in vivo* tumorigenic population was represented by the cells that survived after TiO₂-1 treatment.

We compared biological phenotypes between *in vitro*- and *in vivo*-grown tumor cell lines (Table 5). The *in vitro* cell lines QR/TiO₂-1 and QR/TiO₂-2, derived from QR-32 cells and serially treated with TiO₂-1 and TiO₂-2, respectively, acquired tumorigenicity but not metastatic ability. However, QR/TiO₂-1-P and QR/TiO₂-2-P cell lines, established from the arising tumors after subcutaneous injection of QR/TiO₂-1 and QR/TiO₂-2 respectively, acquired both tumorigenicity and metastatic ability. These results suggest that the titanium dioxides promoted tumorigenic conversion of benign tumor cells, and that metastatic properties were acquired by expanding primary tumors.

Among tumor-derived soluble factors, TGF- β and PGE₂ were increased following conversion of QR-32 cells mediated by TiO₂. In particular, TGF- β , one of the immunosuppressive factors, was increased in QR-32 cells after serial co-culture with TiO₂-1 (QR/TiO₂-1 cell line). This phenomenon correlated with quantitative RT-PCR

results; namely, TiO₂-1 induced TGF- β expression at the implantation site (Figure 2). Although the TiO₂-1-caused production of TGF- β was an intrinsic occurrence, it was not a direct cause for tumor formation because the TGF- β production was not inherited to the tumorigenic cell lines derived from QR/TiO₂-1 cells (Table 5). In our earlier studies, PGE₂, another immunosuppressive mediator, was observed following tumorigenic conversion of QR-32 cells.²⁴ To support it, we confirmed in the current study that PGE₂ production by tumor cells closely correlates with the acquisition of tumorigenic potential (Table 5). Not only our experimental tumor system, but also other tumor models and clinical studies revealed that PGE₂-mediated immunological suppression is one of the mechanisms by which tumor escapes from host immune surveillance, especially in primary tumor development.⁵⁶⁻⁵⁹ However, such host immunity-dependent tumorigenic conversion is not a common feature for human or rodent carcinogenesis since most of their spontaneous tumors are not, or very weakly, immunogenic.^{60,61} Therefore, we realize that it is premature to extrapolate our experimental findings for acceleration of carcinogenic process by the presence of TiO₂ into human being. We need to elucidate whether the phenomenon is universal by using spontaneous or autologous carcinogenesis models.

Recent studies suggested that nano-TiO₂ is presumably harmful to humans.⁵³ Indeed, nano-TiO₂ in sunscreens can catalyze oxidative damage to DNA in cultured human fibroblasts.⁶² More importantly, nano-TiO₂ might be able to enter the human stratum corneum and interact with the immune system,⁴⁹ be translocated to the subepithelial space⁶³ and be subsequently released into the circulation.⁵² The current legislation of nanomaterials is limited, mainly due to the lack of risk information of such a remarkable novelty.⁶⁴ Caution should be taken in their fabrication and handling, and of course, nano-compounds should be disposed of safely.³⁷ These newly synthesized nanomaterials therefore have double-bladed effects; while they can be beneficial to our daily life, they can also have harmful repercussions that were not anticipated at their designing and engineering stages.

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