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Advances in Brief

Induction of Cytotoxicity by Photoexcited TiO_2 Particles[1]

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Abstract

Photoexcited TiO_2 particles can drive various chemical reactions due to their strong oxidizing and reducing ability. To investigate the possible use of this effect for cancer treatment, the antitumor activity of photoexcited TiO_2 particles was studied *in vitro* and *in vivo*. HeLa cells cultured *in vitro* were completely killed in the presence of TiO_2 (50 μg/ml) with 10-min UV irradiation by a 500-W Hg lamp. In contrast, very little cell death was observed from TiO_2 treatment without UV irradiation. Photoexcited TiO_2 particles also significantly suppressed the growth of HeLa cells implanted in nude mice, compared with those receiving TiO_2 alone or UV irradiation alone. The cell death caused by photoexcited TiO_2 particles was significantly protected in the presence of L-tryptophan and catalase. These molecules are quenchers of hydroxyl radicals and scavengers of hydrogen peroxide, respectively, suggesting that the cells were killed by the OH· and H_2O_2 produced from photoexcited TiO_2 particles.

Introduction

When TiO_2 is excited by a photon whose energy is greater than the TiO_2 band gap (3.2 eV), an electron from the valence band can be excited to the conduction band, thus creating an electron-hole pair. The electron and hole can then, if they do not recombine, reduce or oxidize species in the electrolyte solution. For instance, the hole oxidizes a water molecule to yield OH·, and the electron reduces oxygen (O_2) to give superoxide anion (O_2^-) or H_2O_2 (1–3). These reactive oxygen species can drive various chemical reactions. Therefore, TiO_2 particles have been used in design systems for heterogeneous photosynthesis and photocatalysis, and such studies have received commercial attention in the past decade (4–8). However, no attempt has been made in the application of TiO_2 to medicine, for example, as an anticancer modality.

The effect of TiO_2 particles on experimental animals has been investigated from the viewpoint of genetic toxicity. These experiments consistently yield negative results. For example, when TiO_2-coated mica particles were administered to rats in their diet for up to 130 weeks, no significant changes in survival, body weight gains, or hematological or clinical chemistry parameters were observed (9). Similarly, mice receiving a single i.p. injection of TiO_2 particles showed no foreign body reaction to the particles and no carcinogenic potential (10). Thus, TiO_2 particles alone showed no toxicity to experimental animals.

Because of our interest in the effect of chemical reactions of TiO_2 particles produced by UV irradiation, we studied the cell killing effect of TiO_2 particles with and without UV irradiation. A distinct cell killing effect of HeLa cells was observed *in vitro* with photoexcited TiO_2 particles, and tumor growth of HeLa cells was significantly inhibited by the treatment of TiO_2 particles with UV irradiation. Scavengers of hydroxyl radicals and hydrogen peroxide significantly protected the cells from TiO_2/UV toxicity.

Materials and Methods

Cytotoxicity Study. HeLa cells were cultured in a MEM[3] (Gibco) solution supplemented with 10% fetal calf serum in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C.

TiO_2 particles (anatase, p-25; Nippon-Aerosil Co., Tokyo, Japan) with an average diameter of 300 Å, were prepared as described previously (11). The particles were first ultrasonically dispersed in water and then sterilized using an autoclave. TiO_2 particles that aggregated during sterilization were removed by centrifugation (1600 × *g*), and the small TiO_2 particles (0.03–10 μm) in the suspension were collected. The amount of TiO_2 in the suspension was measured with combustion analysis. The TiO_2 aqueous suspension was added to MEM solution supplemented with 10% fetal bovine serum to prepare a MEM solution containing TiO_2 which was used to investigate the cytotoxicity against HeLa cells.

A colony-forming assay (plating efficiency, 80–85%) was used in the *in vitro* study. HeLa cells were plated in 60-mm Petri dishes, and after they were cultured for 5 h at 37°C in CO_2 to allow cell attachment, the old cultured medium was replaced with TiO_2-containing MEM solution. The cells were recultured for 24 h in the dark at 37°C, the TiO_2 solution was removed, and the cells were washed twice with a Hanks' balanced salt solution (Gibco). TiO_2-free medium was finally added to the cells, and the prepared cells were irradiated with a 500-W high-pressure mercury lamp (Ushio Co., Tokyo, Japan) at room temperature. During the irradiation, a water filter was used to remove infrared radiation. After growing in culture again for 10 days, the colonies were fixed with 70% methanol, stained with a 5% Giemsa solution, and counted.

Catalase (Sigma Chemical Company, St. Louis, MO) and L-tryptophan (Tokyo Kasei Company) were dissolved in a PBS solution (pH 7.4) and were filtered through a membrane filter (0.22 μm) for use in scavenger experiments. Either catalase (1, 10, 50, 100 mg/liter) or L-tryptophan (1, 2, 3, 4, 5 mM) were added to the cells during the last 3 h of TiO_2 exposure. After removal of these scavengers, the cells were washed twice with Hanks' balanced salt solution. Irradiation was performed in PBS solution containing the same concentration of the scavenger. During irradiation, a UV pass filter (UVD2, Toshiba Co., Tokyo, Japan) was used to obtain a wavelength between 300 and 400 nm (7–10 J/cm^2). Finally, the cells were cultured in a fresh MEM for colony assay as described above.

Antitumor Effect. HeLa cells subcultured *in vitro* were injected s.c. into the backs of nude mice (2×10^6 cells/mouse). When the tumor became measurable in size (about 2 weeks after inoculation of the cells), the tumor-bearing mice were divided into four groups of five mice each. TiO_2 particles in 0.4 ml of PBS (1 mg TiO_2/ml) containing 5% fetal calf serum were injected into the tumor and surrounding subcutaneous tissue. Three days after the TiO_2 injection, the skin covering the tumor was opened surgically, and the tumor was irradiated directly by the mercury lamp for 1 h (300–400 nm; 14–20 J/cm^2). The skin was then closed. The tumor size was measured at 2- or 3-day intervals. The

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[3] The abbreviations used are: MEM, minimum essential medium; PBS, phosphate-buffered saline.

tumor volume was calculated by using the equation $V = ab^2/2$, where a is the length (mm), b is the width (mm), and V is the volume (mm^3) of the tumor.

Results and Discussion

Cytotoxicity of Photoexcited TiO_2 Particles. The cytotoxicity of TiO_2 (without UV irradiation) was determined by exposing cells to various concentrations of TiO_2 in MEM for 24 h. The surviving fraction of the cells was greater than 90% when the concentration of TiO_2 was in the range of 150 μg/ml, as shown in Fig. 1. When such TiO_2-treated cells were exposed to the light of the 500-W mercury lamp in MEM solution, the cell survival was decreased dramatically, as shown in Fig. 1. For instance, 80% of the cells were killed by 5 min of irradiation in the presence of 50 μg TiO_2/ml, and no cell was viable after 10 min of irradiation.

We also found that TiO_2-treated cells were killed more effectively in PBS than in MEM solution. The difference of cell killing effect was probably due to some UV light being absorbed by components of the MEM. Also, some components of MEM such as mannitol or tryptophan might scavenge the OH· produced from photoexcited TiO_2 particles, resulting in the reduction of cell killing activity.

Antitumor Effect of Photoexcited TiO_2 Particles. The antitumor activity of photoexcited TiO_2 particles was examined against HeLa cells transplanted into nude mice, as shown in Fig. 2. When the tumors were treated with TiO_2 particles alone or with light exposure alone, tumor growth was the same as that of mice without any further treatment. When the tumor was treated with TiO_2 and UV light irradiation at the same time, the growth of tumor was significantly suppressed up to 30 days ($P < 0.05$ by Student's t test). Moreover, when tumors (in another group of mice) treated with TiO_2 and UV light received a second treatment of TiO_2 plus UV light irradiation on day 13, the tumor growth was inhibited more dramatically, as shown in Fig. 2. These facts suggest that the combination of TiO_2 particles and UV light irradiation can suppress tumor growth effectively.

Hyperthermia effect caused by light irradiation is negligible in these experiments. Because the light between 300 and 400 nm was passed through a water filter and a UV passing filter,

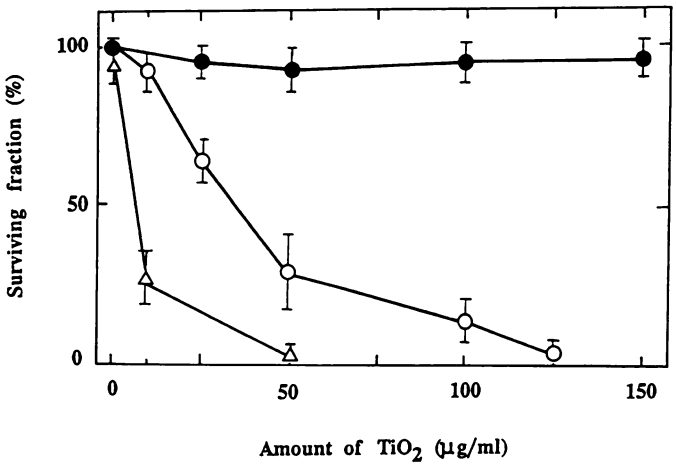

Fig. 1. Surviving fraction of HeLa cells as a function of the amount of TiO_2 particles. After the cells were treated with TiO_2 particles in MEM solution for 24 h, the cells were irradiated with UV light for 5 min (○) or 10 min (△) or received no irradiation (●). In the UV irradiation experiments, cells treated with TiO_2 alone were used as controls. Cultures exposed to TiO_2-free MEM solution were used as controls in experiments without irradiation.

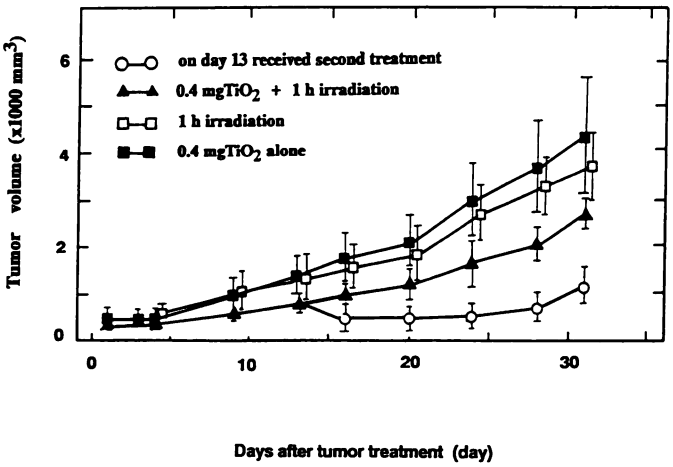

Fig. 2. Antitumor activity of photoexcited TiO_2 particles. Groups of five mice received s.c. inoculations of HeLa cells. Two weeks later, when the tumor became measurable, the tumors received 0.4 mg TiO_2 particles (■), 1 h of filtered UV irradiation (□), or 0.4 mg TiO_2 particles and 1 h of filtered UV irradiation (▲). On day 13, another treatment group received a second treatment of 0.4 mg TiO_2 and 1 h of filtered UV irradiation (○).

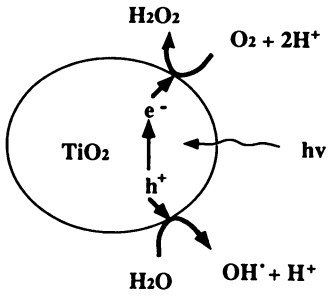

Fig. 3. Schematic illustration of the photocatalytic formation of H_2O_2 and OH· on photoexcited TiO_2 particles.

it did not produce heat at the irradiated site. Since TiO_2 alone had little effect on cell growth *in vitro* and *in vivo*, it is reasonable to conclude that the cell killing effect was caused by TiO_2 particles plus UV irradiation. This cell killing effect can be enhanced effectively by increasing the amount of TiO_2 or light irradiation time, as shown in Fig. 1.

Effect of Scavengers of Reactive Oxygen. It is well known that reactive oxygen species such as OH· and H_2O_2 are formed on photoexcited TiO_2 particles in water solution (1–3). The formation of OH· and H_2O_2 from photoexcited TiO_2 particles is illustrated in Fig. 3. OH· is formed by the oxidation of water via reaction A (1, 12, 13), and H_2O_2 is formed by the reduction of oxygen via reaction B (2, 3, 14, 15).

$$H_2O + H^+ \rightarrow OH\cdot + H^+ \quad \text{(A)}$$

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \quad \text{(B)}$$

Furthermore, some of the H_2O_2 might be cleaved into OH radicals by UV irradiation or by the Fenton reaction (4). These highly oxidizing OH· and H_2O_2 can be expected to be toxic to cells. In order to test this hypothesis, the effects of scavengers of the corresponding reactive oxygen species on cell death were investigated *in vitro*. To prevent the components of the MEM from scavenging the OH· formed on the TiO_2 particles, irradiation was performed in PBS solution. Since the cells were killed more effectively in PBS than in MEM solution, a weak UV light (300–400 nm) obtained by UV pass filter was used

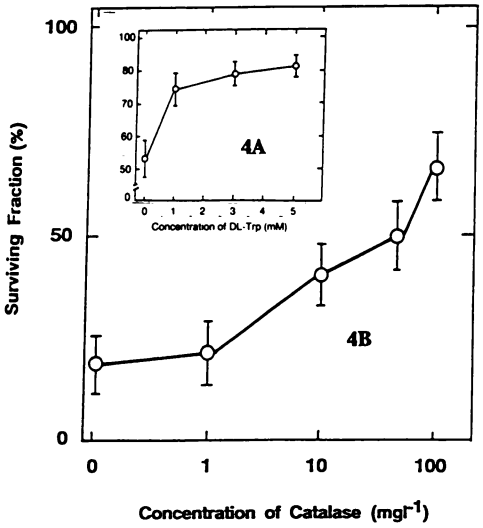

Fig. 4. The effect of catalase or L-tryptophan on the surviving fraction of HeLa cells following treatment with photoexcited TiO_2 particles. After HeLa cells were cultured in 100 μg/ml TiO_2 containing MEM solution for 21 h, each scavenger was added to the cells and incubated for 3 h. The prepared cells were irradiated with filtered UV light as shown in below. *A*, tryptophan and 5 min of UV irradiation; *B*, catalase and 8 min of UV irradiation.

for light irradiation in scavenger experiments.

When the TiO_2-treated cells were irradiated by the filtered UV light in PBS solution, 45% of the cells were killed by photoexcited TiO_2 particles following 5 min of irradiation. In the presence of L-tryptophan, a OH· quencher (16), the cell survival rate was increased. For example, when L-tryptophan was added at 1 and 5 mM, the cell survival rate was increased to 75% and 82%, respectively, as shown in Fig. 4*A*. Since in a control experiment it was found that L-tryptophan alone caused no effect on the cell survival under filtered UV light irradiation, it can be concluded that the OH· produced by photoexcited TiO_2 participates in the process of cell killing.

Catalase, a scavenger of H_2O_2, also protected against cell death caused by photoexcited TiO_2 particles. The relationship between the concentration of catalase and cell survival is shown in Fig. 4*B*. After the HeLa cells were treated with TiO_2 (100 μg/ml) for 24 h, 80% of the cells were killed by 8 min of filtered UV light irradiation in PBS. In the presence of 10, 50, and 100 mg/liter of catalase, the cell survival was increased from 20% to 40%, 50%, and 70%, respectively. Catalase plus light irradiation in the absence of TiO_2 had very little effect on cell viability, suggesting that the increase of cell survival in the presence of catalase is due to catalase decomposition of the reactive H_2O_2 via reaction C.

$$H_2O_2 \xrightarrow{catalase} H_2O + O_2 \qquad (C)$$

The present results showed that under light irradiation, TiO_2 particles had a distinct cell killing effect *in vitro* and a significant antitumor effect *in vivo*, whereas in the dark TiO_2 particles showed no effect on the cell survival *in vitro* and no effect on growth of the tumor *in vivo*. This cytotoxic effect of photoexcited TiO_2 particles is presumed to be due to the generation of strong reactive oxygen such as OH· and H_2O_2 on the surface of TiO_2 under light irradiation. This behavior of TiO_2 suggests the idea of cancer treatment using TiO_2 particles and light irradiation. The cell killing effect of TiO_2 particles under these conditions could be adapted to an anticancer modality by the local or regional administration of TiO_2 particles to the tumor, followed by light irradiation focusing on the tumor. It is reported that the penetration depth of UV light (300–400 nm) into the tissue is limited to a few fractions of a milimeter (17, 18). Although UV light cannot penetrate deeply into the human body, this possible modality could be used for the treatment of superficial tumors in an organ appropriate for light exposure such as skin, oral cavity, trachea, and urinary bladder. The cell killing mechanism of photoexcited TiO_2 particles seems different from the traditional photodynamic therapy using hematoporphyrin. The generation of strong reactive oxygen species on the TiO_2 particles is observed only under UV light. And these reactive oxygen species can permeate into tumor tissue. It may be possible that the modality using TiO_2 particles and UV light produces stronger and more extensive anticancer effects without troublesome side effects such as hypersensitivity to sunlight.

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