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Sonodynamic Therapy using Water-dispersed TiO₂-polyethylene Glycol Compound on Glioma Cells:
Comparison of Cytotoxic Mechanism with Photodynamic Therapy

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Running title: Sonodynamic and photodynamic cytotoxicity by titanium dioxide on glioma

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ABSTRACT

Sonodynamic therapy is expected to be a novel therapeutic strategy for malignant gliomas. The titanium dioxide (TiO₂) nanoparticle, a photosensitizer, can be activated by ultrasound. In this study, by using water-dispersed TiO₂ nanoparticles, an in vitro comparison was made between the photodynamic and sonodynamic damages on U251 human glioblastoma cell lines. Water-dispersed TiO₂ nanoparticles were constructed by the adsorption of chemically modified polyethylene glycole (PEG) on the TiO₂ surface (TiO₂/PEG). To evaluate cytotoxicity, U251 monolayer cells were incubated in culture medium including 100 μg/ml of TiO₂/PEG for three hours and subsequently irradiated by ultraviolet light (5.0mW/cm²) or 1.0 MHz ultrasound (1.0 W/cm²). Cell survival was estimated by MTT assay 24 hours after irradiation. In the presence of TiO₂/PEG, the photodynamic cytotoxic effect was not observed after 20 minutes of an ultraviolet light exposure, while the sonodynamic cytotoxicity effect was almost proportional to the time of sonication. In addition, photodynamic cytotoxicity of TiO₂/PEG was almost completely inhibited by radical scavenger, while suppression of the sonodynamic cytotoxic effect was not significant. Results of various fluorescent stains showed that ultrasound-treated cells lost their viability immediately after irradiation, and cell membranes were especially damaged in comparison with ultraviolet-treated cells. These findings showed a potential application of TiO₂/PEG to sonodynamic therapy as a new treatment of malignant gliomas and suggested that the mechanism of TiO₂/PEG mediated sonodynamic cytotoxicity differs from that of photodynamic cytotoxicity.

Keywords: glioma, fluorescence assay, photodynamic therapy, polyethylene glycol, sonodynamic therapy, water-dispersed titanium dioxide
1. Introduction

Sonodynamic therapy (SDT) for cancer is based on the activation of sonosensitizers by ultrasound (US). This is a new approach for cancer therapy derived from photodynamic therapy (PDT). In the treatment of malignant gliomas, there have been several preliminary studies of PDT using 5-aminolevulinic acid (5-ALA), a porphyrin derivative [1-4]. However, the limited tissue penetrability of light has been a crucial problem [5] because the malignant gliomas often develop in deep brain tissue, such as the basal ganglia or the brainstem. In this regard, US can penetrate deeply into tissues and, moreover, can be focused to the tumor site [6-8]. This indicates that SDT with sonosensitizers is expected to become a novel and effective therapeutic arm. Recently, the effects of sonocatalytic reagents in combination with ultrasound on glioma cells were reported [9, 10]. Some photosensitizers such as porphyrin derivatives demonstrate US-induced cytotoxic reactions [11-18].

Titanium dioxide (TiO2) is also a photosensitizer that has a strong oxidizing activity and produces oxidative radicals under irradiation of ultraviolet (UV) light below 385nm wavelength [19]. The photocatalytic effect of TiO2 has been applied to cancer treatment [20-25], and US irradiation is an alternative energy source for TiO2 [26-28]. Furthermore, toxicological examinations have shown that the TiO2 nanoparticle is basically nontoxic for experimental cells or animals [29-31]. Therefore, we supposed that sonocatalytic TiO2 could become a new material in SDT for various cancers involving malignant glioma.

We have developed a novel water-dispersed TiO2 nanoparticle modified by polyethylene glycole (PEG) and demonstrated its photocatalytic anti-tumor effect on a glioma cell line [32]. These nanoparticle compounds are approximately 50 nm in diameter, and their dispersion and stability in an aqueous medium have been confirmed. These compounds do not cross the blood-brain barrier (BBB) in normal brain. On the other hand, accumulation of the particles in malignant glioma by transvascular delivery is expected because the normal BBB is disturbed in the tumor site [33, 34]. If TiO2/PEG nanoparticles are activated by US irradiation and lead to damage to the glioma cells, SDT using these nanoparticles might be a novel and safe therapeutic strategy for malignant gliomas.

In the present study, the sonocatalytic cell-killing effect of water-dispersed TiO2/PEG nanoparticles on the U251 human glioblastoma cell line was evaluated. Then, the role of oxidative radicals generated from excited TiO2/PEG in tumor cell damage using oxidative radical scavengers was tested, since a previous report speculated that reactive oxidative radicals play a central role in the sonocatalytic process of TiO2, similar to a photocatalytic effect [26]. Furthermore, the differences between photodynamic and
sonodynamic cytotoxicities of this sensitizer were investigated to compare with other reported sensitizers [12, 35, 36].

2. Material and Methods

2.1. Construction of TiO$_2$/PEG

The pH of 100 mg/ml comshaped PEG–maleic acid anhydride (PEGMA, AM1510K; Nihon Yushi Co., Ltd, Tokyo, Japan) dissolved in water was adjusted to 4.0 by 0.1 M NaOH. To activate the carboxyl groups of PEGMA, 0.6 ml of 0.8 mol/l 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (Pierce, Rockford, IL) was added, and the solution was incubated at room temperature for 5 min. Then, 0.3 ml of 0.2 M 4-amino-salicylic acid (4ASA; Wako Jyunyaku, Osaka, Japan) solution was added, and the mixed solution was incubated at 40°C for 16 hrs. Unreacted 4ASA and other small molecules were removed with Amicon Ultra-15 (MWCO = 3000; Millipore, Billerica, MA) by centrifugation five times following the manufacturer’s recommendations. Exchange of the solvent of reacted PEGMA-4ASA was carried out after vacuum drying at 25°C, 20hPa for 3 min and 5hPa for 35 min. The concentration of PEGMA-4ASA was adjusted to 50 mg/ml by dimethylformamide (DMF). An acidic TiO$_2$ sol was prepared by thermal synthesis based on hydrolysis of an organo-titanium compound followed by peptization, as described in detail previously [37]. After hydrolysis of chlorotitanium triisopropoxide (Acros, Morris Plains, NJ), peptization by HNO$_3$ was carried out at 80°C. The reactant was adjusted to a solid content of 20% (wt/vol) with 1.5 M HNO$_3$. After ultrasonication at 200 kHz for 30 min (Midosonic 600; Kaijyo, Tokyo, Japan), particle-size distribution analysis (see “Measurement of particle diameter and zeta-potential” in this section) indicated the presence of approximately 40 nm diameter particles of TiO$_2$ in the solution. The TiO$_2$ solution (0.75ml) was added to 10 ml of DMF, and 5 ml of 50 mg/ml PEGMA-4ASA in DMF was added, followed by stirring. The mixed solution was incubated at 130°C for 16 hrs using Chemist Plaza (Shibata Kagaku, Tokyo, Japan). The reaction was carried out under reflux and vigorous stirring at 600 r.p.m. After the reaction ended, it was cooled to room temperature. Exchange of the solvent of reacted TiO$_2$/PEG was carried out after vacuum drying at 40°C, 5hPa for 10 min. The concentration of TiO$_2$/PEG was adjusted to 10 mg/ml by water. The TiO$_2$/PEG aqueous solution was purified with Amicon Ultra-15 (MWCO = 3000; Millipore) by centrifugation five times following the manufacturer’s recommendations. Its characteristics were similar to those of the TiO$_2$/PAA hybrid [38].
2.2. Cell culture

Human glioblastoma cell line U251 was obtained from ATCC® (the American Type Culture Collection, MD, USA). The cells were cultured in vitro in Dulbecco’s Modified Eagle Medium (DMEM; GIBCO™, NY, USA), containing 10% fetal bovine serum (FBS; GIBCO™), 100 U/ml of penicillin and 100 μg/ml of streptomycin (SIGMA®, MO, USA) and MEM non-essential amino acids (GIBCO™), and maintained at 37°C in a humidified incubator with 5% CO₂ in air.

2.3. UV irradiation

U251 cells (70% confluence) were trypsinized and suspended in DMEM at a concentration of 1×10⁵ cells/ml. The cell suspension (100μl) was plated in each well (1.0×10⁴ cells/well) of a 96-well plate (MICROTEST™96, Becton-Dickinson, NJ, USA) and incubated 24 hrs at 37°C in humidified air with 5% CO₂. Then, the medium was replaced with 100 μg/ml TiO₂/PEG diluted in fresh DMEM without phenol-red and incubated at 37°C for three hours. Phenol red free culture medium was used during UV irradiation to avoid reduction of UV energy as UV penetration is significantly influenced by dyes. After co-incubation, the plates were irradiated with a commercially available UV lamp with a peak wavelength at 360 nm (FL20SS W/18, TOSHIBA, Tokyo, Japan). The light intensity was measured by a coherent power meter (NOVA™, OPHIR, Saitama, Japan). The incident light intensity was 5.0 mW/cm².

2.4. US irradiation

The US apparatus was the commercially available SONIC MASTER ES-2 (OG-Giken, Okayama, Japan). The transducer generates a burst US pulse of 1 MHz center frequency and 100 Hz pulse repetition frequency. The U251 cell suspension (250ml) was dispensed in a 24-well plate coated with collagen I (BD BioCoat™; BD Bioscience, Bedford, MA, USA), and each well contained approximately 2.5×10⁴ cells. Cells were plated, and adjacent wells were not used to avoid the influence of US energy when another well was irradiated. The plates were incubated 24 hrs at 37°C in humidified air with 5% CO₂. Then, the medium was replaced by fresh DMEM containing 100μg/ml of TiO₂/PEG without phenol-red and incubated at 37°C for three hours. For the US alone group, the medium was also replaced with fresh DMEM without TiO₂/PEG and phenol red and incubated for the same amount of time. Phenol red free medium was used in the same conditions for UV and US treatment. The plate was placed 20 mm from the surface of the transducer, and 6% acrylamide gel was buffered between the US transducer and the plate to avoid an increase in temperature. The temperature on the plate was checked using TR-K™ (As One, Osaka,
Japan), and the temperature was kept less than 37°C during irradiation. The transducer generated 50% duty-ratio burst US of 0.4-1.2 W/cm² in reading output power. Then the plates were irradiated by US at room temperature separately for each individual well.

According to the measurements by Feril et al. using the same type of US apparatus [40], the peak acoustic pressure and spatial-average-temporal-average intensity (I_{SATA}) of the apparatus were estimated as 0.795 W/cm² and 0.204 MPa, respectively, at reading output intensity of 1 W/cm² with 50% duty ratio. We also confirmed the attenuation of US by 6% acrylamide gel and a base plate of the plastic well inserted between the transducer surface and cells. The measurements were conducted inside a sufficiently large water tank (0.7×0.5×0.4 m³) to eliminate the effects of standing wave using a membrane hydrophone (HMB-500, Onda, Sunnyvale, CA, USA). The insertions decreased US pressure and intensity to 86.2 ± 4.5% and 74.6 ± 7.8%, respectively. In this paper, US intensities are described using values of reading output unless otherwise indicated.

2.5. Cytotoxic assay (MTT assay)

After UV or US irradiation, the treated cells were stored at 37°C in a humidified incubator with 5% CO₂ in air for 24 hrs. Cell survivability was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [39] per the manufacturer’s instructions (TACSTM, R & D systems, MN, USA). In the UV-treated cells, 10 μl of MTT solution was added to each well, and the plates were incubated at 37°C for 4 hrs. Subsequently, 100 μl of detergent reagents were added to each well to dissolve the formed blue formazan. In the US-treated cells, 25 μl of MTT solution was added to each well, and the plates were incubated at 37°C for 4 hrs, followed by the addition of 250ml of detergent reagents. After the formazan was completely dissolved, the formazan solution was transferred to a 96-well plate. The absorbance of 570 nm for the formazan solution was measured using the SUNRISE™ microplate reader (TECAN, Kawasaki, Japan). Treated and non-treated control wells were compared. The survival rate of the cells was calculated by the following formula:

% Survival rate = \frac{[\text{absorbance of treated group}]}{[\text{absorbance of control group without any treatment}]} \times 100

2.6. Inhibition of cytotoxic oxidative radical

The effect of an oxidative radical scavenger on both UV and US irradiation in the presence of TiO₂/PEG was tested. Glutathione (SIGMA®, MO, USA) was used as a radical scavenger. After 3 hrs incubation of the cells in medium containing 100 μg/ml of
TiO$_2$/PEG with various concentrations of glutathione (10-20mM), the cells were irradiated by UV (5.0mW/cm$^2$, 60 min) or US ($I_{SATA}$; 1.0W/cm$^2$, 50 sec). The treated cells were stored at 37°C for 24 hrs. The cell survivability was assessed by MTT assay.

2.7. Immunofluorescence staining

To assess the viability and damage of U251 cells after UV irradiation for 60 min or US irradiation ($I_{SATA}$; 1.0W/cm$^2$) for 50 sec with 100 μg/ml TiO$_2$/PEG diluted in DMEM, the irradiated cells were stained with Calcein AM/ Ethidium homodimer co-staining kit (Live/Dead Viability/ Cytotoxicity assay kit®, Invitrogen, OR, USA). The cell-permeate esterase substrate Calcein AM is non-fluorescent until enzymatically converted to highly fluorescent calcein (excitation 494nm, emission 517nm), which is retained within live cells. Ethidium homodimer-1 (EthD-1, excitation 528nm, emission 617nm) undergoes fluorescence enhancement upon binding nucleic acids. This dye is excluded from cells that have an intact plasma membrane. On the other hand, they can penetrate the membranes of dead cells and intercalate into nuclear deoxyribonucleic acid (DNA).

For double staining with Calcein AM and EthD-1, after washing the cells with PBS solution on a 24-well plate, PBS containing 2μM of Calcein AM and 4μM of EthD-1 was added to each well, and cells were incubated for 30 min at room temperature in the dark. Cells were washed twice by PBS and observed using fluorescent microscopy (IX71, U-LH100HGPO, Olympus, Tokyo, Japan).

To assess the cell damage mechanism by UV and US irradiation, the treated cells were stained with Annexin V-FITC and Propidium iodide (PI) (Annexin V-FITC Apoptosis Kit, BioVision, CA, USA). The acute phase of apoptosis is accompanied by translocation of phospholipids, which are normally restricted to the inner surface of the cell membrane, to the outer leaflet of the still intact cell membrane, where they can be recognized by Annexin V (excitation 490 nm, emission 518 nm). The nucleus is stained with PI (excitation 536 nm, emission 620 nm), which penetrates the damaged cell membrane. U251 cells, which were treated with UV for 60 min or US for 50 sec with 100 μg/ml TiO$_2$/PEG or without TiO$_2$/PEG in a 24-well plate, were washed twice with PBS, followed by 500 μl of cold binding buffer containing 5 μl Annexin V-FITC and 5 μl PI, and were incubated for 15 min at room temperature in the dark. Cells were washed twice and then evaluated with fluorescent microscopy. This double staining procedure (Annexin V-FITC and PI) was performed 6 hrs after irradiation in order to assess the pattern of cell damage. For quantification of the staining pattern, the percentages of stained cells (Annexin V-FITC only, PI only, and both) were determined in high power field images (x 400) created by digitally merging fluorescence images.
and phase-contrast images.

Further, the US-treated and UV-treated cell membranes were labeled by Vybrant® Dil (excitation 549 nm, emission 565 nm, Lonza, MD, USA) for assessment of the sonotoxic damage of the cell membrane. Vybrant® Dil has the highly lipophilic nature of carbocyanine dyes and stains viable cell membranes. Cell nuclei were labeled with Hoechst 33342 (excitation 350 nm, emission 461 nm, Invitrogen), which can bind DNA regardless of cell survival. Immediately after US or UV irradiation, treated U251 cells were washed twice with PBS, and then the cells were incubated in DMEM containing 50 μM Vybrant® Dil at 37°C for 10 min. After rinsing with PBS, the cell nuclei were labeled by Hoechst 33342 (0.1 mg/ml final concentration in PBS) at room temperature for 10 minutes and observed with fluorescent microscopy.

2.8. Statistical analysis

Data in figures is expressed as mean ± standard deviation. Statistical analysis was performed with Statcel2 software (OMS publishing Inc, Saitama, Japan). Differences between the groups were considered significant when P-values of one-way analysis of variance (ANOVA) followed by Bonferroni test for the post hoc determination of significant differences were less than 0.01.

3. Results

3.1. Sonodynamic antitumor effect

The cytotoxicity of TiO₂/PEG induced by various intensities of US (I_{SATA}; 0.4-1.2 W/cm², 30 sec) was tested on U251 cells by MTT assay (Fig. 1). Minor cytotoxicity at high US intensity was observed even without TiO₂/PEG in the medium. In the presence of 100 μg/ml TiO₂/PEG, the cell survival rate remarkably decreased depending on US intensity. TiO₂/PEG itself (without US exposure) showed no cytotoxic effect on U251 tumor cells (data not shown).

3.2. Comparison between sonodynamic and photodynamic effect of TiO₂/PEG on cell survival

Fig. 2 shows the influences of irradiation time on survival rate of the treated U251 cells. Throughout this comparison, the intensity (I_{SATA}) of US irradiation was fixed at 1.0 W/cm² because our first experiment showed that the difference of survival rates in the absence and presence of TiO₂/PEG was largest at this setting (Fig. 1). Cytotoxicity was not confirmed in the group that underwent UV irradiation alone (Fig. 2a). In the US
irradiation group, a slight suppression was seen in the group that underwent US exposure even without TiO$_2$/PEG, and the sonocatalytic cytotoxic effect remarkably increased with TiO$_2$/PEG. In the presence of TiO$_2$/PEG, the photodynamic cytotoxic effect was not observed until the UV light dose exceeded 6.0 J/cm$^2$ (UV irradiation time; 20 min), while the sonodynamic cytotoxicity effect was almost proportional to the duration of US irradiation. The surviving fraction was reduced to about 10% after 60 min of UV irradiation time and 50 sec of US irradiation time, respectively.

3.3. The inhibiting effects of oxidative radical scavenger

To investigate the influences of oxidative radicals for cytotoxicity induced by UV irradiation and US irradiation in the presence of 100 $\mu$g/ml TiO$_2$/PEG, the effect of 10-20 mM of glutathione, an active oxidative radical scavenger, on cell survival was tested. Figure 3 demonstrates cell survival rate at 60 minutes after UV irradiation and at 50 seconds after US irradiation ($I_{SATA}$; 1.0 W/cm$^2$). In the UV irradiation group, the cell damages were obviously inhibited by glutathione (Fig. 3a, ## p<0.01)). In the US irradiation group, glutathione did not inhibit cell killing in comparison to the control (Fig. 3b, ** p<0.01). On the other hand, glutathione relatively inhibited cell killing at 20 mM compared to 0 mM (Fig. 3b, ## p<0.01). These findings suggested that the influence of reactive oxidative radicals in the cell damage mechanism of SDT might be different from PDT.

3.4. The differences of mechanism in anti-tumor activities between sonodynamic treatment (SDT) and photodynamic treatment (PDT)

To investigate the mechanism of U251 cell damage by PDT and SDT with TiO$_2$/PEG, UV- or US-treated cells were stained with Calcein (green) and EthD-1 (red) to distinguish between viable cells and dead cells in the presence of 100 $\mu$g/ml TiO$_2$/PEG (Fig.4). Immediately after treatment, minimal cytotoxicity was observed in UV-treated cells, while approximately more than half of US-treated cells were stained with EthD-1. Twenty-four hours after treatment, almost all treated cells in both groups were negative for Calcein.

In addition, the cell death mechanism was tested by double staining with Annexin V-FITC and PI using fluorescent microscopy six hours after irradiation (Fig. 5 and 6). In the absence of TiO$_2$/PEG, several US-treated cells were positive for PI (less than 20%) although there were scarcely any fluorescence signals from the UV-treated cells. In the presence of TiO$_2$/PEG, the cell membranes were clearly stained with Annexin V-FITC and the nucleus was also stained with PI, implying apoptotic changes of these cells in
both UV-treated and US-treated cells. More than 50% of UV-treated cells were stained with both PI and Annexin V-FITC, indicating that the pathway of cell death was predominantly apoptosis. On the other hand, approximately 40% of US-treated cells were stained only with PI, suggesting that death is not only the result of apoptosis (Fig. 6).

Furthermore, the damage of cell membrane by US exposure in the presence and absence of TiO$_2$/PEG was analyzed by Vybrant® Dil staining (Fig. 7). Immediately after UV irradiation, the cell membrane with cellular processes was labeled by Vybrant® Dil regardless of whether TiO$_2$/PEG was present or absent (Fig. 7, upper). In the ultrasound alone group (without TiO$_2$/PEG), the cell membrane also maintained viability. On the other hand, the fluorescence signals decreased or disappeared immediately after US exposure in the presence of TiO$_2$/PEG (Fig. 7, lower). This result also demonstrated that the damage of cell membrane by TiO$_2$/PEG mediated SDT was completed quickly.

4. Discussion

First, results achieved in this study strongly suggested that TiO$_2$/PEG could be a novel sensitizer for SDT, because cell damage induced by ultrasonic treatment was remarkably enhanced by TiO$_2$/PEG in vitro.

Furthermore, we attempted to compare TiO$_2$/PEG mediated SDT and PDT. The output irradiation intensity was set at 1.0 W/cm$^2$ (reading output power), for 50 seconds because the approximately 90% cell killing effect (measured by MTT assay) at this level of US energy corresponded to the UV exposure experiment (approximately 90% cell killing effect with 5.0 mW/cm$^2$ for 60 minutes). However, with US irradiation, the buffer (6% acrylamide gel) was inserted between the US transducer and the well plate to avoid an increase in temperature of the culture medium. It is assumed that actual energy to the glioma cells on the well plate was reduced.

According to the measurement using the membrane hydrophone, insertion of the buffer gel and the base plate of the well decreased US intensities applied to glioma cells to 74.6%. Therefore, the US intensity of 1 W/cm$^2$ in reading output is estimated to be 0.59 W/cm$^2$ in $I_{SATA}$ in the condition with no standing wave generation. In the practical experimental condition, however, strong reflection at a water-air interface and well walls causes generation of a standing wave inside the well, resulting in enhanced effects of US exposure. Although it is difficult to predict intensity distributions in the plastic wells with standing wave, it is important to determine whether inertial cavitation was produced. Feril et al. investigated US-induced apoptosis with the same type of
ultrasonic apparatus (SONIC MASTER ES-2, OG-Giken) and a similar US exposure configuration using a culture dish [40]. They evaluated US-induced free radicals (hydroxyl radical) by electron paramagnetic resonance (EPR) analysis and confirmed the presence of free radicals at intensities above 0.3 W/cm$^2$ with 10% duty ratio ($I_{SATA}$: 0.081 W/cm$^2$) and increases with increasing intensity. Based on their experiment, the effective US intensities applied to cells in our sonodynamic treatment condition should be above the threshold for inertial cavitation, accounting for the decline of US intensity by the buffer gel and the base plate of the well.

Since a direct comparison of PDT and SDT is difficult, this study demonstrated one aspect of various conditions. Nevertheless, this kind of comparison is still important and is the first step for clinical development of SDT in glioma therapy as many photosensitizers such as porphyrin derivatives have been converted into sonosensitizers [11-18].

The sonodynamic cytotoxicity on U251 cells was almost proportional to the duration of US exposure. In contrast, photodynamic cytotoxicity seemed to have a threshold UV energy dose. Kessel et al. compared the effects of photodynamic and sonodynamic cytotoxicity mediated by mesoporphyrin. They found differences in cytotoxic activity between photo and US treatments [36], and their result was basically identical to ours. They suspected that the threshold in the photodynamic cytotoxic effect may indicate the capacity for limited repair of photodamage, and US-induced loss of viability results from rapid cell destruction and is proportional to the time of US exposure. In distinguishing the mechanisms of PDT and SDT, formation of a singlet oxygen has been one of the most important issues. In the present study, glutathione, a singlet oxygen scavenger, almost completely inhibited photodynamic cytotoxicity of TiO$_2$/PEG. Reactive oxidizing radicals, such as hydroxyl radicals, superoxide anion and singlet oxygen are formed on photocatalytic TiO$_2$ in the aqueous environment [19, 20, 23, 41]. These oxidative radicals are expected to be toxic to the tumor cells [42]. The inhibition of photodynamic cytotoxicity by glutathione indicated that oxidative radicals might play a primary role in cell damage in TiO$_2$/PEG mediated PDT.

On the other hand, with glutathione, the inhibition of sonodynamic cytotoxicity with TiO$_2$/PEG was limited. In recent years, the mechanism of sonodynamic therapy using sonosensitizers was not only due to an oxidizing chain reaction of cell membranes and cellular components by formation of oxidative radicals but also physical destabilization of the cell membrane by sonosensitizers. Thus, the cell was more susceptible to shear forces [35, 43]. Our results suggested a series of oxidizing chain reactions by oxidative radicals was not a dominant cytotoxic mechanism of sonocatalytic therapy for glioma.
cells using TiO$_2$/PEG. The fluorescence assays for cells treated by UV or US with TiO$_2$/PEG clearly indicated that the TiO$_2$/PEG mediated sonodynamic cytotoxic pattern is different from the photodynamic pattern. Staining with EthD-1 revealed that the majority of cells were damaged by TiO$_2$/PEG mediated SDT immediately after treatment, although in PDT groups, there were hardly any damaged cells. Further, TiO$_2$/PEG was expected to enhance rapid cell damage because only a fraction of cells treated by US alone (without TiO$_2$/PEG) were stained with EthD-1 (data not shown). Similarly, the fluorescence staining with Vybrant® Dil, which labels viable cell membrane, showed that US with TiO$_2$/PEG crucially damaged the cell membrane immediately after irradiation, whereas the viability of the cell membrane was preserved immediately after US irradiation without TiO$_2$/PEG. These results indicated that TiO$_2$/PEG mediated SDT can rapidly damage glioma cells by injury to the cell membranes. Generally, the most sensitive structures in the cell are biomembranes, and the mechanism of SDT-induced cell damage was mainly due to mechanical stress leading to physical disruption of the cell membrane by sensitizers such as porphyrin and rhodamine derivatives in the close vicinity of cells and cavitation bubbles [35]. It is also suspected that US irradiation with TiO$_2$ nanoparticles results in destruction of the secondary structure of protein molecules followed by loss of the space configuration, and the alteration of the configurations might cause forfeiture of their function [28]. In addition, the effect of SDT may depend on subcellular and intracellular localization of sonosensitizers. Hydrophilicity can be an important property of cell permeability of sensitizers [13], and TiO$_2$/PEG nanoparticles have colloidal stabilization and dispersibility in aqueous solution. Our previous investigations have shown that these nanoparticles might attach to tumor cell membranes and be incorporated in tumor cells [32]. This experimental result indicated that the water dispersed TiO$_2$/PEG exists in close vicinity of the cell membrane. This property results in physical disruption of cell membranes by sonocatalytic TiO$_2$/PEG and indicates that this nanoparticle might be suitable for sonodynamic therapy.

In both UV and US in the presence of TiO$_2$/PEG, cell staining with Annexin V-FITC occurred 6 hrs after treatment, suggesting that apoptosis had been induced. However, the radical scavenger was not able to significantly suppress cytotoxicity in SDT using TiO$_2$/PEG in contrast with PDT. Based on these results, although oxidative radicals generated from TiO$_2$/PEG by US irradiation might induce apoptosis in SDT, which is the same as PDT, it was inferred that apoptotic cell death through oxidative radicals in SDT might be less dominant in comparison with PDT.

In conclusion, the sonodynamic antitumor effect was strongly enhanced by
water-dispersed TiO$_2$/PEG, and we confirmed that the mechanism of sonodynamic cytotoxicity differed from that of photodynamic cytotoxicity. TiO$_2$/PEG nanoparticles are approximately 50 nm in diameter. Particles of this size cannot cross the blood-brain barrier (BBB) theoretically [44]. However, these nanoparticles should accumulate in malignant glioma by transvascular delivery because the BBB is disrupted in malignant glioma [33, 34, 44]. Thus, TiO$_2$/PEG nanoparticles should be distributed only within the malignant glioma. In addition, magnetic resonance imaging-guided high intensity focus ultrasound for brain tumors has been developed in recent years [6-8]. By combining this method with accumulation of TiO$_2$/PEG, it may be possible to relieve damage to adjacent unaffected brain tissue. Although further investigations are needed, TiO$_2$/PEG nanoparticles can be a novel clinical material for sonodynamic therapy for malignant glioma.

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 References


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**Figure Captions**

**Figure 1**
The surviving fraction of U251 cells as a function of US intensity with or without TiO₂/PEG diluted in DMEM. Cell survival rate was measured by MTT assay 24 hours after US irradiation. US frequency was 1 MHz with 50% duty ratio, and irradiation time was 30 seconds. Bars in the figure represent average ± standard deviations.

**Figure 2**
The surviving fraction of U251 cells as a function of UV (a) and US (b) irradiation time. The cells were treated with or without TiO₂/PEG. The concentration of TiO₂/PEG was 100 μg/ml. UV light intensity was 5.0 mW/cm², and US intensity was 1.0 W/cm² in reading output.

**Figure 3**
The effect of a radical scavenger (glutathione) on cytotoxicity 60 minutes after UV irradiation (a) and 50 minutes after US irradiation (b). The concentration of TiO₂/PEG was 100 μg/ml. The data are expressed as the mean ± standard deviation (n=10-12), and statistical analysis was performed using one-way ANOVA followed by Bonferroni test. ** shows a significant difference (p<0.01) between the control (TiO₂(-) and glutathione(-)) and each treatment. ## shows a significant difference (p<0.01) between the non glutathione (TiO₂(+) and glutathione(-)) group and the glutathione-treatment group (TiO₂(+) and glutathione(+)) . Cell damage in PDT was completely inhibited by both concentrations of glutathione (a), while the scavenger did not reduce cell death induced by SDT (b).

**Figure 4**
Double immunostaining for Calcein (green) and Ethidium homodimer-1 (red) of treated cells with TiO₂/PEG for 60 minutes UV irradiation (upper) and 50 seconds US irradiation (lower). The concentration of TiO₂/PEG was 100 μg/ml. Immediately after treatment, UV-treated cells were stained with Calcein, while many US-treated cells were already stained with Ethidium homodimer-1 (left). Twenty-four hours after treatment, almost all treated cells were not stained with Calcein in both PDT and SDT groups (right).

**Figure 5**
The figures show the phase-contrast (upper), fluorescent (2nd and 3rd lines) and merged (lower) microscopic images of the PDT and SDT cells 6 hours after irradiation. The cells were stained with Annexin V-FITC (green) and Propidium iodide (PI) (red). “TiO$_2$(-)” means irradiation in the absence of TiO$_2$/PEG, and “TiO$_2$(+)” means irradiation using the culture medium containing 100 μg/ml TiO$_2$/PEG. In the TiO$_2$(-) group, several US-treated cells were stained with only PI. In both PDT and SDT with the TiO$_2$/PEG, the cell membranes were clearly stained with Annexin V-FITC, and the nuclei were also stained with PI.

**Figure 6**
The percentages of cells stained with Annexin V-FITC alone (ANN-V), Propidium iodide alone (PI) and both in each treatment 6 hours after irradiation. The staining patterns of treated cells were determined by digitally-merged fluorescent and phase-contrast images. In the absence of TiO$_2$/PEG, UV-treated cells were hardly stained by Annexin V-FITC, and about 20% of US-treated cells were stained with only PI. In the presence of TiO$_2$/PEG, more than 50% of UV-treated cells were concomitantly stained with PI and Annexin V-FITC, while US-treated cells were predominantly stained with PI alone.

**Figure 7**
The fluorescent micrographs demonstrate damage of the cell membrane immediately after UV irradiation (upper) and US irradiation (lower). The viable cell membranes were stained with Vybrant® Dil (red), and cell nuclei were stained with Hoechst 33342 (blue). The signals of Vybrant® Dil remarkably decreased after US exposure in the presence of TiO$_2$/PEG (lower right).
Figure 2

(a) PDT

(b) SDT

Survival (%) vs. UV irradiation time (min) for different concentrations of TiO₂:

- TiO₂ (-)
- TiO₂ 100 μg/ml

Survival (%) vs. US irradiation time (sec) for different concentrations of TiO₂:

- TiO₂ (-)
- TiO₂ 100 μg/ml
Figure 4

PDT 0hr 24hr

SDT

200μm
Figure 5

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Annexin V-FITC

PI

merge
Figure 7

TiO$_2$/PEG free

TiO$_2$/PEG 100 μg/ml

PDT

SDT

200μm