Anti-Tumor Effects of Liposome-Encapsulated Titanium Dioxide in Nude Mice

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Key Words

Apoptosis · Bladder cancer · Liposome · Oxidative stress · TiO₂

Abstract

Anatase particles of titanium dioxide (TiO₂) absorb ultraviolet (UV) light which is shorter than 415 nm. Photoexcited TiO₂, a strong oxidizer, is expected to inhibit malignant cell growth. Liposomes accelerate endophagocytosis to the cytoplasm of encapsulated materials. In this study, we examined anti-tumor effects of TiO₂ and liposome-encapsulated TiO₂ (LT) with UVA irradiation by an air pouch cancer model using NBT-II bladder cancer cells, which simulates bladder cancer. Injection of TiO₂ or LT into the air pouch was followed by UVA irradiation via the opened pouch. Tumors of TiO₂ + UVA and LT + UVA groups showed more pronounced necrotic areas, apoptotic indices, nitrotyrosine formation, tumor growth inhibition and increased survival compared with control groups. Especially the LT + UVA group showed more remarkable anti-tumoral effects than the TiO₂ + UVA group, which was associated with higher TiO₂ incorporation. These findings suggest that LT might be more effective than noncoated TiO₂ in the treatment of bladder cancer.

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Introduction

Titanium dioxide (TiO₂) is a semiconductor. When anatase-type TiO₂ particles absorb ultraviolet (UV) light shorter than 415 nm (3.0 eV) corresponding to the band gap of TiO₂, it creates an electron hole pair [1]. Two mechanisms of the TiO₂ photocatalyst reaction have been proposed [2]. One is the direct action of photoexcited TiO₂ on cells. Photoexcited TiO₂ is a strong oxidizer, and it is expected that the cells are directly oxidized by the holes formed in the balance band. The other proposed process is the indirect action. Hydroxyl radicals (OH·) and/or perhydroxy radicals (HO₂·) generated from H₂O may attack proteins and lipids in the cell membrane and cellular components. Kubota et al. [3, 4] and Gai et al. [3, 4] reported that strong oxidation by TiO₂ inhibits malignant cell growth. In an in vivo study, TiO₂ particles alone showed no toxicity to experimental animals [3].

In Japan, approximately 5,000 patients die from bladder cancer every year [5]. The intravesical cavity can be easily observed through an endoscope. Cancer in the vesicular cavity can be treated with superficial application of TiO₂ combined with UV irradiation. In this study, we used an air pouch model formed in mouse subcutaneous tissue to obtain an animal model simulating bladder cancer, i.e. cancer in a cavity. We investigated the anti-tumoral effect of the photocatalytic activity of TiO₂ + UVA in comparison with liposome-encapsulated TiO₂ (LT). Thus, bladder cancer is an adequate model for photodynamic therapy (PDT).

Abbreviations: LT = liposome-encapsulated TiO₂; PBS = phosphate-buffered saline; PDT = photodynamic therapy; UV = ultraviolet.

Table 1. Necrotic and apoptotic effects of LT

Group	Necrotic area, %	Apoptotic index, %	Nitrotyrosine concentration, nM
PBS	13 ± 2 ^{a, b}	2 ± 1 ^{a, b}	43 ± 12 ^{c, d}
LT	14 ± 2	3 ± 1	47 ± 14
TiO ₂ + UVA	45 ± 6 ^{a, c}	14 ± 3 ^{a, e}	471 ± 97 ^{a, c}
LT + UVA	77 ± 10 ^{b, c}	22 ± 4 ^{b, e}	971 ± 289 ^{a, d}

^ap < 0.0005; ^bp < 0.0001; ^cp < 0.001, ^dp < 0.005; ^ep < 0.01, calculated by Mann-Whitney test.

Materials and Methods

Cell Culture

A rat urinary bladder cancer cell line, NBT-II (Dainihon, Tokyo, Japan), was grown in E-MEM (Sigma, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂ in air.

Preparation of TiO₂ and LT

TiO₂ particles (anatase, AMT-100, Teika, Tokyo, Japan; mean diameter: 6 nm) were suspended in PBS, ultrasonically dispersed and sterilized using an autoclave. TiO₂ particles 100 and 50 nm in diameter were hardly incorporated into liposomes. The TiO₂ aqueous suspension (2 ml) was added to empty liposome preparations (EL-C-01, DPPC: 26 mM, Nippon-Oil & Fats, Tokyo, Japan). The amount of TiO₂ particles in the suspension was measured with an inductively coupled plasma atomic emission spectrometer (ICPS-1000 III, Shimadzu, Kyoto, Japan). Treatment solutions were prepared to contain 400 µg/ml of TiO₂. The dosage of TiO₂ is set to be 400 µg for each tumor.

UV Irradiation

UVA irradiation (peak length 365 nm; 5 J/cm²) was performed with an UV lamp (xx-15 BLB; UVP, Tokyo, Japan). UVB and UVC with wavelengths <330 nm were cut with an UV cut filter (UV-33, Toshiba, Tokyo, Japan).

Anti-Tumor Effects in an Air Pouch Model in Nude Mice

Thirty male BALB/c nu/nu mice (SLC Japan, Shizuoka, Japan), 4 weeks old, were inoculated with a single-cell suspension of 5 × 10⁶ NBT-II cells with 0.2 ml PBS into the subscapular subcutaneous tissue. When tumors reached 5 mm in diameter, air (3 ml) was injected into the subcutaneous tissue surrounding the tumor to form an air space. These mice were then subjected to different treatments: PBS alone (PBS group), LT without UVA irradiation (LT group), TiO₂ with UVA (TiO₂ + UVA group) and LT with UVA (LT + UVA group). To examine the direct effects of these substances, TiO₂ or LT solutions were injected into the subcutaneous air pouch. After 3 h of incubation, the pouch was opened in order to expose the tumor surface. The tumor surface was irradiated with UVA, and the skin incision was closed. Two mice from each group were sacrificed 24 h after treatment for histological examination. Six other mice per group were monitored for tumor growth and survival.

Histological Examination

Thin sections (4 µm) of the archival specimens were immunostained with anti-single-strand DNA antibody (IBL, Takasaki, Japan) to detect single-strand DNA breaks in cells caused by free radicals produced through UVA-excited TiO₂ [6]. Tissue image of hematoxylin-eosin staining was captured into computer and necrotic area was marked and calculated by NIH image (National Institutes of Health, Bethesda, Md., USA). Cells positive for anti-single-strand DNA antibody per 500 cells were counted using a microscope.

Enzyme-Linked Immunosorbent Assay (ELISA) for Nitrotyrosine

ELISA for anti-nitrotyrosine antibody (Hycult Biotechnology, Uden, The Netherlands) was performed according to the manufacturer's instructions for 3 times to detect oxidative stress [7, 8]. Whole lysates were extracted from liquid nitrogen-frozen tumor materials according to the method described previously [9].

Detection of Tissue-Incorporated TiO₂

Thin sections of the tumor archival specimen were dewaxed and hydrated with PBS and solubilized by boiling with 5 M acid guanidine sulfate for 10 min. TiO₂ suspended in 5 M acid guanidine sulfate-containing bovine serum albumin (1 µg/µl, Sigma, St. Louis, Mo., USA) was used as a control. Tissue extracts (20 µg/20 µl) and TiO₂ suspension (20 µl) were put into a 48-well dish and irradiated with UVA (10 J/cm²) followed by addition of methanol (80 µl). Then 100 µg of DPPP (diphenyl-1-pyrenyl phosphine) in 100 µl chloroform (Dojindo, Kumamoto, Japan) were mixed with samples, and fluorescence intensity (λ 380 nm) was measured.

Statistical Analyses

Statistical significance was examined by the unpaired Mann-Whitney test (nonparametric difference), the Kaplan-Meier model (survival curve), the Cox proportional hazard model (survivals) and Spearman's rank test (nonparametric correlation; Statview 4.5, Abacus Concepts, Berkeley, Calif., USA). Statistical significance was defined as a two-sided p value <0.05.

Results

Necrotic and Apoptotic Effects of TiO₂ and LT with UVA Irradiation

To confirm anti-tumor effects of TiO₂ and LT, the subcutaneous tumors were analyzed histologically in each group 24 h after treatment. Firstly, necrotic areas

Fig. 1. Necrotic and apoptotic effect of TiO₂ or LT. **a-f** Histology of tumors treated with PBS (**a, d**), TiO₂ + UVA (**b, e**), LT + UVA (**c, f**). **a, c, e** H & E staining. **b, d, f** Necrotic areas (8, 33, and 84%, respectively) are shown in black. Bar = 200 µm. **g-j** Single-strand DNA-positive apoptotic cells were detected in 6, 4, 16 and 32% cells in tumors of PBS (**g**), LT (**h**), TiO₂ + UVA (**i**) and LT + UVA (**j**) groups, respectively. Bar = 100 µm.

Fig. 2. Incorporation of TiO_2 aggregates in tumor tissue. Tissue incorporation of TiO_2 was examined by UVA-evoked TiO_2 -derived hydroperoxide detected by DPPH fluorescence intensity. **a** Standard curve between TiO_2 and generated hydroperoxide. **b** TiO_2 content in tumor tissue treated with PBS, TiO_2 + UVA (T + UVA) and LT + UVA ($p < 0.005$, vs. LT + UVA). Bars show standard deviations.

Fig. 3. Tumor growth and survival in the PBS, TiO_2 + UVA (T + UVA) and LT + UVA groups. **a** Mean diameter (mm) of the tumors. Bars indicate standard deviations. **b** Kaplan-Meier analysis of survival. * $p < 0.005$.

and apoptotic indices were examined in the tumors (fig. 1a–f, table 1). Tumors treated with TiO_2 + UVA or LT + UVA showed large necrotic areas at the surface and internal regions of the tumors (fig. 1a–f). TiO_2 + UVA and LT + UVA groups demonstrated a more pronounced necrotic effect than PBS or LT groups ($p < 0.0005$, and $p < 0.0001$, respectively). LT + UVA treatment yielded higher necrotic effects than TiO_2 + UVA treatment ($p < 0.0001$). In the periphery of the necrotic areas, single-strand DNA-positive apoptotic cells were detected (fig. 1g–j, table 1). PBS and LT groups showed low apoptotic indices. TiO_2 + UVA and LT + UVA groups showed apoptosis in 14 ± 3 and $22 \pm 4\%$ cells in the tumors, respectively, which were higher than those in PBS and LT groups ($p < 0.0005$ and $p < 0.0001$, respectively). LT + UVA showed higher apoptotic effects than TiO_2 + UVA ($p < 0.001$).

Oxidative Stress by TiO_2 and LT with UVA Irradiation

Oxidative stress generated by TiO_2 + UVA or LT + UVA in tumors was examined by nitrotyrosine formation, i.e. major oxidative products [7, 8] (table 1). Nitrotyrosine concentrations in TiO_2 + UVA or LT + UVA groups were 471 ± 97 and 971 ± 289 nM, respectively, which were higher than those in PBS and LT groups ($p < 0.001$, and $p < 0.005$, respectively). LT + UVA generated more nitrotyrosine than TiO_2 + UVA ($p < 0.0005$). Nitrotyrosine concentration was correlated with the necrotic area and apoptotic index ($p = 0.0001$ and $p = 0.0002$, respectively).

Incorporation of TiO_2 in Tumors

To confirm incorporation of TiO_2 in the tumor tissue, in vitro hydroperoxide generation by UVA-excited TiO_2 was examined (fig. 2). A standard curve showed good

Table 2. Correlation between TiO₂ incorporation and necrosis, apoptosis, nitrotyrosine concentration and the 50% survival period

	S-test	95% CI	p value
Necrotic area	0.6144	0.3175–0.8790	0.0015
Apoptotic index	0.5877	0.1518–0.8322	0.0103
Nitrotyrosine concentration	0.6144	0.1923–0.8446	0.0067
50% survival period	0.6295	0.2160–0.8516	0.0051

Nonparametric correlation was calculated by Spearman rank test (S-test). CI = Confidence interval.

correlation between TiO₂ content and hydroperoxide production (fig. 2a). In tumor tissue, equivalent TiO₂ content to hydroperoxide generation was significantly higher in the LT + UVA group than that in the TiO₂ + UVA group (fig. 2b, $p < 0.005$). TiO₂ incorporation was correlated with necrotic area, apoptotic index and nitrotyrosine concentration (table 2, $p = 0.0015$, $p = 0.0103$, and $p = 0.0067$, respectively).

Tumor Growth and Survival in Mice Treated with TiO₂ and LT with UVA Irradiation

Tumor growth and survival were examined (fig. 3). The tumors in TiO₂ + UVA and LT + UVA groups showed slower growth in comparison with tumors in the PBS group (TiO₂ + UVA vs. PBS; $p < 0.005$, LT + UVA vs. PBS; $p < 0.001$). Survival in TiO₂ + UVA and LT + UVA mice was longer than in PBS mice (TiO₂ + UVA vs. PBS; $p < 0.005$, LT + UVA vs. PBS; $p < 0.001$). Fifty percent survival periods were 44, 73 and 83 days in PBS, TiO₂ + UVA, and LT + UVA, respectively. LT + UVA treatment yielded slower tumor growth and longer survival than TiO₂ + UVA treatment ($p < 0.01$). Fifty percent survival periods of all mice were significantly correlated with TiO₂ incorporation (table 2, $p = 0.0051$).

Discussion

PDT is one of the local treatments with selective anti-tumor effects. Photosensitizers, oncotropic optical photoreactive chemical compounds and low-output lasers are used for PDT. Recently, similar to urological cancers, good results of PDT have been reported for the intravesical 5-aminolevulinic acid treatment [10]. We examined the efficacy of TiO₂ and LT as photosensitizers in the present study.

To confirm the anti-tumoral effect of the TiO₂ photocatalyst reaction, we compared TiO₂ tissue incorporation with tumor necrosis, apoptosis and nitrotyrosine formation. The TiO₂ photocatalyst reaction induces oxidation of cellular components, which results in both necrosis and apoptosis [11]. TiO₂ incorporation was correlated with the necrotic area, apoptotic index, nitrotyrosine concentration and 50% survival periods. Nitrotyrosine is an oxidative product of tyrosine reacting with nitric peroxide [7]. PBS and LT without UVA resulted in minimal nitrotyrosine levels. In contrast, nitrotyrosine was generated in tumors of TiO₂ + UVA or LT + UVA groups, which was closely associated with TiO₂ incorporation. These results suggest that UVA-evoked TiO₂-dependent oxidative stress is responsible for the anti-cancer effect of TiO₂.

TiO₂ incorporation into cells depends on phagocytosis, and the incorporated TiO₂ is metabolized by macrophages [3]. LT facilitated incorporation into tumor tissue, which was expected to enhance intracellular TiO₂ concentration. Comparing LT and TiO₂, LT + UVA groups showed 2.5 times higher TiO₂ incorporation in tumor tissue than TiO₂ + UVA groups. Because liposomes are rapidly internalized into cells, LT particles are more effectively transferred to the target cells than noncoated TiO₂.

Liposomes have been employed as drug carriers in vitro and in vivo studies [12, 13]. Four mechanisms are proposed for the interaction of liposomes with cells [14]: (1) stable adsorption – the association between intact liposomes and cell surfaces without entering the cell, (2) endocytosis – intact liposomes delivered to endocytotic vesicles, (3) lipid transfer – lipid exchange between cells and liposomes without the cell interacting with the aqueous content of the liposome and (4) fusion – the liposome bilayer fuses with the cell plasma membrane depositing the contents of the liposome into the cytoplasmic space. In this study, liposomes might suggest two more advantages: firstly, polar photosensitizer can pass the cellular membrane barrier when encapsulated within liposomes, and secondly, water-insoluble photosensitizer can be administered to the cell. In the in vitro treatment, cationic liposomes showed growth inhibition in NBT-II cells (data not shown). However, cytotoxicity was negligible in animals. Excess liposomes may be washed out from the stromal tissue by the blood circulation.

In conclusion, our data suggest adjuvant therapy in bladder cancer as a possible treatment option. A combination of transurethral resection and endoscopic TiO₂-liposome PDT might be beneficial in the treatment of superficial and invasive bladder cancer.

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