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The study of the photokilling effect and mechanism of ultrafine TiO_2 particles on U937 cells

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Abstract

The killing effect of photoexcited ultrafine TiO_2 particles (UFP TiO_2) on U937 cells has been studied. The mechanism concerned has been further investigated by DNA agarose gel electrophoresis. The electrophorogram shows that reactive oxygen species produced by photoexcited UFP TiO_2 can damage DNA, which results in cell death. Finally, a new assumption of the way to kill cancer cells is proposed. © 1997 Elsevier Science S.A.

Keywords: Ultrafine TiO_2 particles; Photocatalytic killing effect; U937 cells; Reactive oxygen species

1. Introduction

When a TiO_2 semiconductor absorbs light with energy greater than its band gap, electrons in the valence band are excited to the conduction band, creating electron–hole pairs. Such photogenerated holes have strong oxidizability and can cause various chemical reactions such as photoreduction, photocatalysis and photo-organic synthesis [1–5]. In recent years, in contrast to the many studies using TiO_2 particles for photodecomposition of organic pollutants, few studies have investigated the application of TiO_2 to biology, especially in the field of cancer treatment. Usually, the way to treat cancer includes radiation therapy and chemical therapy by using antitumor drugs, which may generate wide-ranging side-effects in the body. In this study human U937 monocytic leukemia cells were used as experimental objects. We investigated the photocatalytic killing effect of ultrafine TiO_2 particles (UFP TiO_2) on malignant cells, observed the changes in DNA induced by photoexcited TiO_2 and studied the mechanism of action of photoexcited TiO_2 on malignant cells.

2. Experiment

2.1. Preparation of TiO_2 colloid solution

TiO_2 colloid solutions were prepared [6,7] by hydrolysis of tetrabutyl titanate, $Ti(OC_4H_9)_4$, as follows: 25 ml of $Ti(OC_4H_9)_4$ was added to 4 ml of isopropanol. Then the mixture was added to 150 ml of distilled deionized water containing 2 ml of 70% nitric acid, keeping stirring for 6 h at ≈ 75 °C. Approximately 150 ml of TiO_2 colloidal solution which can be stable for several months at 4 °C were obtained after removing the organic layer. The size of the colloidal particles determined by transmission electron microscope (TEM) was about 10 nm. The morphology of the TiO_2 particles was examined with an atomic force microscope.

The pH value of the TiO_2 colloid solutions used in the subsequent experiment had to be adjusted from 1.8 to 5.5–6.5 in order not to damage the normal growth of cells. Therefore, a 1 mol l^{-1} NaOH aqueous solution and a polymer stabilizer, e.g. polyvinyl alcohol, were added to the colloid solution in order to prevent TiO_2 particles from precipitating. The final TiO_2 colloid solutions were sterilized by autoclaving and diluted to the required concentration.

2.2. Cell culture and UFP TiO_2 treatment

Human U937 monocytic leukemia cells were kindly provided by the National Laboratory of Pharmaceutical Technology, Nanjing University, China. The cells were maintained at 37 °C in RPMI 1640 supplemented with 10% fetal bovine serum and 100 μg ml^{-1} of kanamycin (growth medium) in a humidified atmosphere of 5% CO_2. All experiments were performed using cells during the exponential growth phase. Cell concentration was determined by using a

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hemocytometer and the cell density was adjusted to the required final concentration ($\approx 10^6$ cells ml^{-1}).

U937 cells ($\approx 10^6$ cells ml^{-1}) were treated with TiO_2 diluted in RPMI 1640 medium for 2 h at 37 °C. Then the solutions were irradiated with a 500 W high pressure Hg lamp at room temperature. A UV pass filter was used to obtain a light wavelength between 300–400 nm. The light intensity at the liquid surface was measured by a radiometer–photometer.

We arranged three groups for experiments. One group was treated in the absence of either TiO_2 or light. Another was treated with TiO_2 and different catalase concentrations irradiated by UV light. In the third groups, we substitute L-cystenine for catalase.

2.3. Measurement of the viability of U937 cells

Samples of the solution were done at requisite time intervals by pipetting 100 μl from the beakers into wells of a 24-well plate. Viable cells in the samples were measured by using the MTT (tetrazolium) staining method [8]. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in PBS at 5 mg ml^{-1} and filtered to sterilize it. At the times indicated below, 20 μl of stock MTT solution was added to all wells of an assay, and plates were incubated at 37 °C for 4 h. Acid–isopropanol (100 μl of 0.04 mol l^{-1} HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the blue–violet crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plate were read on a Microelisa reader, using a test wavelength of 570 nm, taking the solution without MTT as the comparative sample; then the optical absorptions $[A]$ were obtained. Plates were normally read within 1 h of adding the isopropanol. The survival rate can be calculated according to $[A]_t/[A]_i$ where $[A]_i$ is the optical absorption of untreated cells.

2.4. Analysis of DNA agarose gel electrophoresis

Untreated or TiO_2-treated cells were collected by centrifugation. Then the cell pellets ($(1–2) \times 10^6$ cells) were resuspended in 40 μl of phosphate–citrate (PC) buffer, consisting of 192 parts of 0.2 mol l^{-1} Na_2HPO_4 and 8 parts of 0.1 mol l^{-1} citric acid (pH 7.8), at room temperature, for at least 30 min. After centrifugation at 1000*g* for 5 min, the supernatant was transferred to 0.5 ml Eppendorf tubes and concentrated by vacuum evaporation for 20 min. A 3 μl of 0.25% Nonidet NP-40 in distilled water was then added, followed by 3 μl of a solution of RNase A (1 mg ml^{-1}, also in water). After 30 min incubation at 37 °C, 3 μl of a solution of proteinase K (1 mg ml^{-1}) was added and the extract was incubated for an additional 30 min at 37 °C. After the incubation, 12 μl of loading buffer (0.25% bromophenol blue, 40% sucrose) was added and the entire content of the tube was transferred to the gel. Horizontal electrophoresis of 0.8% agarose gel with 5 μg ml^{-1} of ethidium bromide was performed at 25 V for 10 h. The DNA in the gel was visualized under UV light and photographed [9,10].

2.5. Hydroxylated reaction of benzoic acid with photoexicited TiO_2

The system consisting of 0.305 g benzoic acid, 10 ml of 0.20 mol l^{-1} NaOH aqueous solution and 15 ml 0.8 g l^{-1} TiO_2 colloid solution was irradiated for 72 h. The reaction mixture, which had a small amount of brownish resinous products, was extracted with carbon tetrachloride after being acidified to pH 1–2 with 1 mol l^{-1} HCl to remove colored products into the aqueous layer. The organic layer was extracted with 5% sodium bicarbonate, and then the aqueous layer, adjusted to pH 5, was examined by the spectrofluorometer RF-5000.

3. Results

3.1. Effect of photoexcited TiO_2 on U937 cells

The fact that the surviving fraction was greater than 90% after 30 min, as shown in Fig. 1(a), indicates that UFP TiO_2 without light irradiation showed little toxicity to living cells. The effects of UV light without TiO_2 are shown in Fig. 1(b) with the surviving fraction of U937 cells given as a function of the light irradiation time. About 20% of cells were killed after a 30 min exposure, whereas after a 20 min exposure, more than 90% of the cells survived. Once TiO_2 was added, the U937 cells were killed at a much higher rate as shown in Fig. 1(c). For example, in the presence of 1000 μg ml^{-1} of TiO_2, 80% of the cells were killed after 10 min of UV light irradiation, and after 30 min irradiation, all cells were killed. Therefore, it is concluded that photoexcited UFP TiO_2 has an active killing effect on U937 cells.

3.2. The changes in U937 cells induced by photoexcited UFP TiO_2

3.2.1. Morphological changes in U937 cells

Untreated and TiO_2-treated cells were collected by centrifugation and were then resuspended in 10% trichloroacetic acid. The samples were pipetted into 24-well plate which was

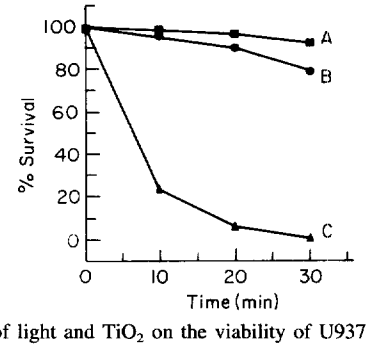


Fig. 1. Effect of light and TiO_2 on the viability of U937 cells: (a) TiO_2 (1000 μg ml^{-1}) in the dark; (b) no TiO_2 in the light; (c) TiO_2 (1000 μg ml^{-1}) in the light.

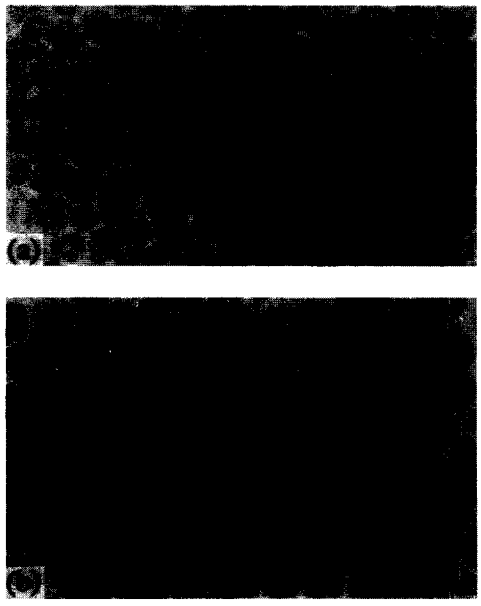


Fig. 2. Morphological changes in U937 cells induced by photoexcited UFP TiO_2: (a) without treatment; (b) after treatment with photoexcited UFP TiO_2.

directly observed with an inverted phase-contrast microscope. Two photos were shown in Fig. 2. We found that after treated by photoexcited TiO_2, the cellular shape was condensed, nucleus was dispersed in fragments and some apoptotic body appeared as well.

3.2.2. *The changes of DNA gel electrophoresis in U937 cells*

DNA molecules carry negative changes in neutral pH solution, so they move to the positive electrode in an electric field through a gel medium which possesses the property of a molecular sieve. The larger the DNA molecules are, the larger is the friction drag and the harder it is for the DNA molecules to move through the pores in the gel. The shifting rate of DNA in gel medium is in inverse proportion to the logarithm of the number of base pairs. In other words, because of different DNA molecular weights, the final location of DNA after electrophoresis will be different. From Fig. 3, the fact that the shifting rates of DNA in lanes 2, 3 and 4 were faster than that in lanes 1 indicated that base pairs in DNA of U937 cells treated with photoexcited TiO_2 were damaged, which led to the rupture of the DNA chain and the decrease of molecular weight. Furthermore, the cellular genomic DNA showed a characterization of DNA ladder after electrophoresis, and on the background of the DNA ladder there are some free radical-destroyed DNA fragments. So it may be concluded that the death of U937 cells was due to the damage of DNA, and 1000 $\mu g\ ml^{-1}$ of TiO_2 with irradiation had a remarkable effect on cells shown in the electrophorogram.

3.3. *Effect of photoexcited TiO_2 with catalase on U937 cells*

Catalase is a scavenger of H_2O_2, which specifically decomposes H_2O_2 into H_2O and O_2. Fig. 4 indicates that in the

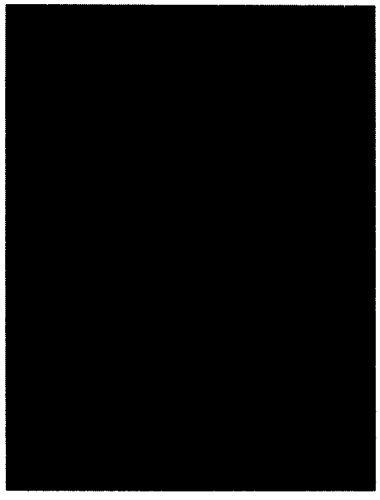


Fig. 3. DNA gel electrophorogram of untreated and treated cells. U937 cells were treated with 0 (lanes 1), 1000 (lanes 2), 500 (lanes 3) and 200 (lanes 4) $\mu g\ ml^{-1}$ of photoexcited TiO_2 for 1 h.

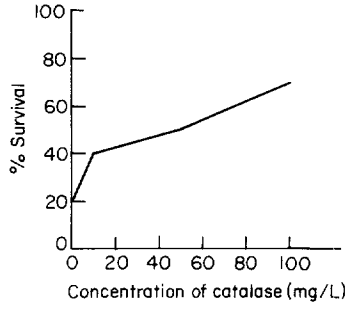


Fig. 4. Effect of photoexcited TiO_2 on the survival of U937 cells in the presence of catalyse. UV radiation time, 15 min; TiO_2 concentration, $0.5\ g\ l^{-1}$.

presence of 10, 50 and 100 $mg\ l^{-1}$ of catalase, the cell survival was increased from 20% to 40%, 50% and 70%, respectively, and catalase plus light irradiation in the absence of TiO_2 had very little effect on cell viability. These results suggest that the increase of cell survival in the presence of catalase is due to decomposition of the reactive H_2O_2, which also indicates that in the photoexcited TiO_2 system, H_2O_2 is playing a role in photokilling U937 cells.

3.4. *Effect of photoexcited TiO_2 with L-cysteine on U937 cells*

In the presence of L-cysteine, a $^{\cdot}OH$ quencher, the cell survival rate was increased. Fig. 5 shows that when L-cysteine was added at 1 and 5 $mmol\ l^{-1}$, the cell survival increased to 75 and 82%, respectively. Since in a control experiment it was found that L-cysteine alone caused no effect on the cell survival under filtered UV light irradiation, it can be concluded that the $^{\cdot}OH$ was produced by photoexcited TiO_2 participates in the process of cell killing.

3.5. *Detection of hydroxylated production of benzoic acid*

A fluorimetric technique was used to identify and determine any hydroxylated benzoates. After hydroxylated reac-

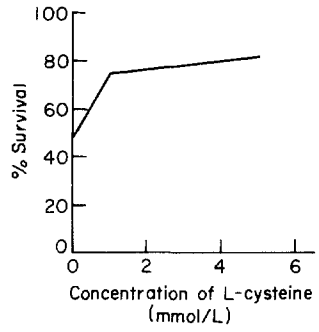


Fig. 5. Effect of photoexcited TiO_2 on the survival of U937 cells in the presence of L-cysteine. UV radiation time, 15 min; TiO_2 concentration, 0.5 g l^{-1}.

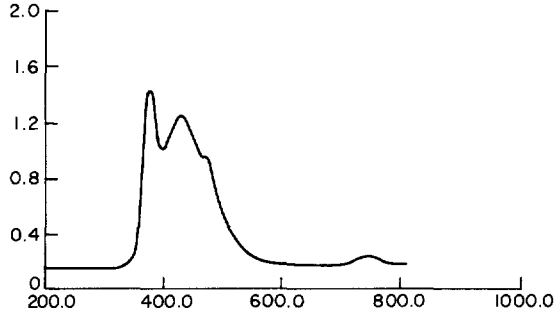
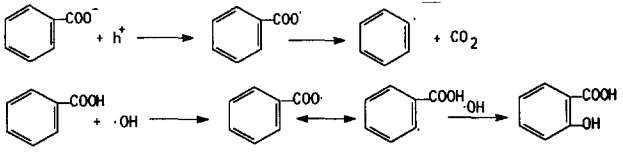


Fig. 6. Fluorescence spectrum of TiO_2 with benzoic acid after 72 h of UV irradiation and purification.

tion of benzoic acid with photoexcited TiO_2, the reaction mixture, after removal of the brownish resinous products, showed two emission peaks at 380.8 nm and 424.0 nm (Fig. 6). The positions of the peaks and the shapes of the spectra are identical with those of standard benzoic acid solutions and salicylic acid solutions, respectively. The detection of salicylic acid in the experiments suggest that the attack on the parent benzoic acid by $^{\bullet}OH$ radicals induced from photoexcite TiO_2 with water is a likely route. The following reactions appear reasonable for the photooxidation of benzoic acid.

$$TiO_2 + h\nu \rightarrow h^+ + e^-$$

$$H_2O + h^+ \rightarrow \cdot OH + H^+$$



4. Discussion

As a semiconductor, TiO_2 possesses good photocatalytic activity. When a TiO_2 particle absorbs light with energy greater than its band gap (3.2 eV), electrons in the valence band are excited to the conduction band, creating electron–hole pairs. Under the action of electric field in space charge layer, electrons, apart from holes, transfer to the surface of TiO_2 particles. When the photogenerated holes react with water attached on TiO_2 and when the photogenerated electrons react with oxygen in water, the reactive oxygen species (ROS) such as $^{\bullet}OH$, $HO_2^{\bullet}$, H_2O_2 are produced as follows:

$$TiO_2 + h\nu \rightarrow h^+ + e^- \quad (1)$$

$$H_2O + h^+ \rightarrow {}^{\bullet}OH + H^+ \quad (2)$$

$$O_2 + e^- \rightarrow O_2^{-\bullet} + H^+ \rightarrow HO_2^{\bullet} \quad (3)$$

$$2HO_2^{\bullet} \rightarrow O_2 + H_2O_2 \quad (4)$$

$$H_2O_2 + O_2^{-\bullet} \rightarrow {}^{\bullet}OH + OH^- + O_2 \quad (5)$$

Bard et al. [11] have detected free-radical intermediates ($^{\bullet}OH$, $HO_2^{\bullet}$) in the photodecomposition of water at TiO_2 particulate systems with spin trapping and electron spin resonance techniques. We have also experimentally proved that ROS such as $^{\bullet}OH$, H_2O_2 are present in photoexcited TiO_2 particulate systems. These ROS will easily react with biomolecules, which results in the damage of organism structure through a series of peroxidative chain reaction and even can damage the DNA, as indicated by DNA gel electrophoresis.

Human U937 monocytic leukemia cells treated with photoexcited UFP TiO_2 were effectively damaged, and they showed the characteristics of apoptosis such as membrane blebbing and DNA fragmentation, especially the DNA ladder. Apoptosis, also called programmed cell death, is a different process of cell death from necrosis. A number of cancer cells have been reported to undergo apoptosis when cells were treated with antitumor agents, radiation or high temperature. In this experiment, it was ROS such as $^{\bullet}OH$, $HO_2^{\bullet}$, H_2O_2 that resulted in apoptosis.

In this experiment, the ultrafine TiO_2 particulate system was used, yielding various advantages: (1) because ultrafine TiO_2 particles yield an extremely large surface area, higher reaction rates can be expected; (2) particles of TiO_2 can be incorporated by the living cells.

Results from this study show the remarkable killing effect of photoexcited UFP TiO_2 on leukemia cells. ROS, such as $^{\bullet}OH$, H_2O_2, play an important role in this kind of killing process. We can assume that the method used in this experiment is also suitable to kill other kinds of cancer cells because ROS have no selectivity as to substrate. This assumption will be a new avenue to treat cancer. Although light (300–400 nm) used in this study cannot penetrate the human body deeply, this possible modality could be used for the treatment of superficial tumors in an organ appropriate for light exposure such as the skin and oral cavity trachea. If a fiber-transmitted laser is used as a light source, the fiber can be inserted into subcutaneous tumor tissues, and irradiation of light can be carried out frequently without surgical opening of the skin. Hence, it could be possible to apply this modality to several tumors of other organs.

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