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Killing of cancer cell line by photoexcitation of folic acid-modified titanium dioxide nanoparticles

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ABSTRACT

Folic acid (FA) was coupled onto titanium dioxide nanoparticles to provide a specificity of cell targeting. Modification was confirmed by infrared absorption spectra and zeta potential that suggested a formation of linkage between carboxylic acid of FA and titanium dioxide. The surface attachment of folic acid did not cause a significantly change in particles size, but led to a reduction of photocatalytic activity of TiO_2. The FA-modified TiO_2 nanoparticles could be internalized by cells at a much faster rate than the unmodified TiO_2, due to the mediation of folate receptor on the cancer cells. The UV irradiation caused death of HeLa cells pretreated with FA-modified TiO_2 more effectively than that of HeLa cells treated with unmodified TiO_2. Results suggest that to modify TiO_2 with folic acid using appropriately the FA-to-TiO_2 mass ratio of 0.2 could yield nanoparticles having higher cytotoxicity under photoexcitation. Photocatalytic TiO_2 nanoparticles could not only damage to cellular components including plasma membranes leading to cell nucrosis but also induce the programming cell death. Results from flow cytometry-based analysis indicated that the mechanism of cell death was a combination of necrosis and apoptosis.

1. Introduction

The photocatalysis of titanium dioxide (TiO_2) has been investigated for decades since Fujishima and Honda published their findings in 1972 [1]. Upon UV radiation, TiO_2 particles can generate active free radicals ($OH^\bullet$ and O_2^-) that are responsible of decomposing organics on the particle surface. Because of this photocatalytic property, titanium dioxide has been widely used for decomposition of organic pollutants [2–5]. In addition to the finding by Matsunaga et al. that microbial cells can be killed by the photocatalysis of titanium dioxide [6,7], numerous papers have been published on using this semiconductor photocatalyst for disinfection and killing of bacteria, viruses, fungi, and even cancer cells [8,9]. Although the biocide activity and induced cytotoxicity by photoexcited TiO_2 particles is well reported, the mechanism underlying the cell-killing process remains largely unknown. On the bactericidal activity, Maness et al. proposed that TiO_2 promotes peroxidation of the polyunsaturated phospholipid component of the lipid membrane and induce a major disorder in the *Escherichia coli* cell membrane, which subsequently results in the loss of cell viability [10]. Since TiO_2 particles are subject to uptake by cell via phagocytosis, cells are damaged also from the inside of cytoplasm [11]. In the present paper, we attempt to reveal the mechanism and cell-killing effect of photoexcited TiO_2 nanoparticles modified with folic acid.

Folic acid (FA) has been used for drug targeting to cancer cells since the folate receptor is significantly overexpressed on the surface of human cancer cells [12,13]. The folate receptor-mediated drug delivery is based on the conjugation of drug with folic acid, which is internalized by folate receptor-mediated endocytosis. Furthermore, the attachment of drug to folic acid does not normally interferer with the binding of folate for its receptor. Folic acid has been immobilized on superparamagnetic magnetite [14], polymer nanoparticles [15], or incorporated to a dendrimer-based therapeutic nanodevice [16] for tumor cell-selective targeting. In the present study, folic acid was coupled on the surface of TiO_2 for the selective binding to cancer cells.

2. Experimental

2.1. Modification of TiO_2 with folic acid

The TiO_2 used in this study was Degussa P-25, obtained as a gift from Degussa, Taiwan Branch. A specified amount of folic acid (Sigma) was dissolved in 0.1 M sodium hydrogen carbonate ($NaHCO_3$) solution adjusted to pH 5.5 with HCl and NaOH. TiO_2 nanoparticles were dispersed in deionized water to a concentration of 0.1 g/ml by sonication for 10 min. The average particle diameter of TiO_2 prior to modification was estimated to be ca. 28 nm on the basis

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of TEM image. The hydrodynamic diameter for Degussa P-25 TiO_2, however, was subject to change by the dose concentration in the culture medium as indicated in the literature [17]. The TiO_2 dispersion then added slowly to a folic acid solution with a volume ratio of 1:9, and the resultant mixture was stirred for reaction for one day. The reaction mixture was finally dialyzed against 1 mM $NaHCO_3$ solution for one day to remove unreacted folic acid, yielding folic acid-modified TiO_2 particles. It is noted that the whole process for the preparation and preservation of modified TiO_2 should be kept in dark conditions.

The photocatalytic activity was characterized by the rate constant for the degradation of methylene blue (Riedel-deHaën) [18]. The rate constant (k, s^{-1}) was evaluated from the plot of $\ln(C_{A0}/C_A)$ vs. irradiation time (t), where C_{A0} and C_A are the concentrations of methylene blue at the initial time and at time t, respectively. The concentration of methylene blue was determined by evaluating the absorbance at 664 nm.

The zeta potential of unmodified and folic acid-modified TiO_2 was determined using Malvern Zetasizer-3000 and samples were buffered with 1 mM NaCl and adjusted to different pH values with NaOH and HCl. For FT-IR analysis, the sample was mixed with KBr (1:10, w/w), pressed into a pellet and placed into the infrared spectrometer. Unmodified and folic acid-modified TiO_2 nanoparticles were also characterized by transmission electron microscopy (TEM) on Hitachi H600.

2.2. Cellular uptake of unmodified and FA-modified TiO_2

HeLa cells were cultured in the MEM medium containing non-essential amino acids solution, sodium pyruvate, and Penicillin/Fungizone/Streptomycin (all medium components were obtained from Sigma). Cells were seeded on a 35 mm dish (1×10^6/dish) for one day for cell adhesion. A dispersion of unmodified or folic acid-modified TiO_2 (200 μg/ml in MEM medium) was then applied to the HeLa cells for culture for 5 min, 30 min, 1 h, 2 h or 6 h. The uptake of TiO_2 by cells was characterized by using flow cytometry to evaluate the intensity of side angle scatter (SSC). Chinese hamster ovary (CHO) cells, as the control of non-folate receptor expression cells [19], were also cultured with TiO_2 particles for 30 min and cellular uptake of TiO_2 particles was estimated by the same method for comparison.

2.3. Photokilling study

Cells were seeded onto a 24-well multiplate (8×10^4/well) for culture for one day. After washing with PBS buffer, cells were incubated with a dispersion of TiO_2 in MEM medium for 24 h or folic acid-modified TiO_2 for 30 min for cellular uptake. After a wash, cells were mixed with 0.5 ml PBS and irradiated with UV at a distance of 1 cm from the 100 W long-wave ultraviolet lamp (Blak-Ray model B 100AP; UVP, Upland, CA) for 5 min, 15 min or 30 min. Cells were then subjected to viability assay.

The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method, which was based on measuring the activity of living cells via mitochondrial dehydrogenases. Cells were washed with PBS buffer and then incubated with MTT (Sigma) solution (0.5 mg MTT/ml in PBS) at 37 °C for 4 h. After incubation, an aliquot of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to dissolve the resulted formazan crystals. Afterward, the product was quantified by measuring absorbance at 570 nm. The MTT stock solution (0.5 mg MTT/ml PBS) was stored at 4 °C in dark not longer than 2 weeks and filtered with a 0.22 μm filter prior to use. The cell survival was defined as the ratio of the viability of treated cells and that of non-treated control (neither TiO_2 treatment nor UV illumination).

Cells after incubation with TiO_2 and UV illumination were characterized by flow cytometry using Annexin-V-FITC and propidium iodide (PI) labeling. The staining solution was prepared by mixing 20 μl Annexin-V-FITC labeling reagent and 20 μl PI into 1 ml Annexin V binding buffer (all from Strong Biotech Corp). Cells released from 35 mm dish by using trypsin-EDTA were mixed with 3× MEM medium, centrifuged, and washed with PBS buffer. The cell pallet was then suspended in 100 μl of the staining solution and the resulting suspension was allowed for incubation for 10–15 min in dark at the room temperature. Finally, the stained cell suspension was added with 0.8 ml PBS buffer and subjected to flow cytometry analysis.

3. Results

3.1. Modification of TiO_2 with folic acid

Folic acid is a water-soluble vitamin B9 and has a pI value of 5.3. Since the pI value of TiO_2 is ca. 6, the modification of folic acid on TiO_2 was then carried out at a pH between 5.3 and 6 in order to enhance the interaction between folic acid and TiO_2. The conjugation of folic acid and TiO_2 was achieved simply by incubation at pH 5.5 in dark conditions for 1 day. As expected, the zeta potential of TiO_2 decreased with pH and its value switched from positive to negative around pH 6, as shown in Fig. 1. When an FA-to-TiO_2 mass ratio of 1 was used, the zeta potential curve for modified TiO_2 particles shifted significantly to the left. Increasing the FA-to-TiO_2 ratio to 5, the left shift of zeta potential curve was more severe. If the FA-to-TiO_2 ratio increased to 10.3, however, the curve was not very different from that for the FA-to-TiO_2 ratio of 5. These results suggest that there was a saturation of functionality on TiO_2 for complexion with folic acid.

Infrared absorption spectra of unmodified and folic acid-modified TiO_2 are shown in Fig. 2. The characteristic peak around 1637 cm^{-1} for TiO2, which is the characteristic peak for banding vibration of water molecule adsorbed on TiO_2, disappeared after the modification with folic acid. Also, the stretching vibration of OH groups of water molecules adsorbed on the unmodified TiO_2 was observed. The water molecules adsorbed on the (1 1 1) face of anatase TiO_2 containing five coordinate Ti(IV) atoms characterize an IR peak at ~3200 cm^{-1} [20]. But this wide and strong peak was smoothed out after coupling with folic acid. After the adsorption of folic acid, the appearance of bands due to asymmetric and symmetric stretching vibrations of carboxylate salt (–COOM) peaks (1512 cm^{-1} and 1440 cm^{-1}) suggested a formation of linkage between carboxylic acid of FA and titanium atom. The free car-

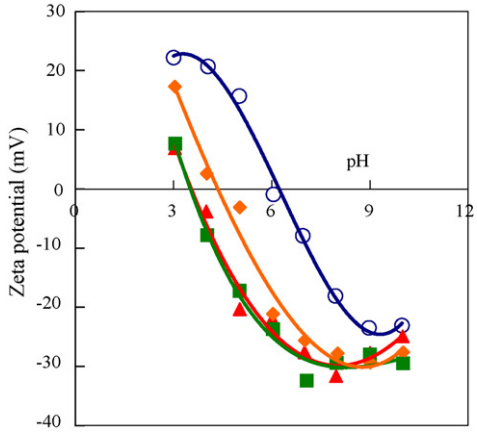

Fig. 1. Zeta potential of unmodified (○) and modified titanium dioxide with folic acid using FA-to-TiO_2 ratios of 1 (♦), 5 (■), and 10.3 (▲).