Phagocytosis of Titanium Dioxide Particles and Titanium Dioxide Particles Chemically Modified by Hematoporphyrin

Ruxiong CAI, Hideki SAKAI, Kazuhito HASHIMOTO, Yoshinobu KUBOTA[†]
and Akira FUJISHIMA*

Received October 23, 1991 ; Accepted December 4 1991

Hematoporphyrin (Hp) was chemically linked to TiO₂ particles, and phagocytosis of the TiO₂-Hp particles in cultured HeLa cells was compared with that of unmodified TiO₂ particles by transmission electron microscope and fluorescence microscope measurements. When cultured HeLa cells were treated with the TiO₂-Hp particles for 3 h, some of them were phagocytized into cytoplasm, whereas the unmodified TiO₂ particles were not. On the other hand, after 24 h treatment, both the TiO₂-Hp and the unmodified TiO₂ particles were phagocytized. On the basis of these experimental observations, it was concluded the TiO₂-Hp particles are phagocytized more easily than the unmodified TiO₂ particles.

1 INTRODUCTION

When TiO₂-coated mica were administered to male and female Fischer 344 rats, they induced no consistent or biological changes in survival, body weight gains, hematologic or clinical chemistry parameters¹⁾. A long-term dietary administration of up to 5% TiO₂ to Fischer 344 rats and B6C3F1 mice did not significantly show toxicity and incidence of tumors²⁾. Similarly, mice receiving a single intraperitoneal injection of TiO₂ particles showed no foreign body reaction to the particles, and no carcinogenicity³⁾. Thus, all studies showed that TiO₂ particles have no toxicity to experimental animals. Recently, studies in vitro of cultured cells also showed that TiO₂ ultrafine particles have little cytotoxicity⁴⁾.

On the other hand, when TiO₂ is irradiated with a light whose energy is greater than the band gap of TiO₂, it has a strong oxidation and reduction power to drive various chemical

reactions^{5,6)}. Using this photo-effect, we have recently applied photoexcited TiO₂ electrode and TiO₂ powder to kill cancerous HeLa cells^{4,7)}. In the particulate system, we found that in the presence of TiO₂ powder, HeLa cells were completely killed with UV irradiation, but were all viable without irradiation. These results suggest that the large effect of "photokilling" would be applied to destroy tumor by injecting TiO₂ into the tumor and irradiating it. The studies also showed that TiO₂ particles were phagocytized into cytoplasm following treatment with TiO₂ particles for 24 h⁴⁾. Such phagocytized TiO₂ particles are considered to kill the cells effectively. Therefore enhancing the efficiency of phagocytosis is one of the factors to get larger ability in "photokilling".

Here, the character of TiO₂ particles surface was altered by chemical modification. The surface of TiO₂ particles was modified with hematoporphyrin (Hp), whose derivative is known to be localized in tumor selectively, in order to increase the efficiency of phagocytosis. It was confirmed with a Fourier transform infrared spectrometer (FT-IR) that Hp was chemically bound to TiO₂ particles. Then, the TiO₂-Hp particles were exposed to the culture cells for

Department of Synthetic Chemistry, The University of Tokyo (Hongo, Bunkyo-ku, Tokyo 113, Japan)

[†] Faculty of Medicine, Yokohama City University (Fukuura, Kanazawa-ku, Yokohama 236, Japan)

Key Words: Phagocytosis, Chemical Modification, Cultured Cells, TiO₂ Particles, Hematoporphyrin

various treatment time, and the phagocytized particles in the cells were detected by a transmission electron microscope (TEM). The efficiency of phagocytosis was compared with that of unmodified TiO_2 particles. Since TiO_2 -Hp particles emits red fluorescence, TiO_2 -Hp phagocytized by the cells was also observed with a fluorescence microscope.

2 MATERIALS AND METHODS

2.1 TiO_2 and TiO_2 -Hp particles

TiO_2 particles (anatase, P-25; Nippon-Aerosil Co. Tokyo, Japan) with an average size of 300 Å in diameter were used. Hp, 3-(2-aminoethylpropyl) trimethylsilane and dicyclohexylcarbodiimide (DCC) and the other reagents were obtained from Kanto Kagaku Co. Tokyo, Japan. The modification of TiO_2 particles with Hp was carried out according to the following procedure. First TiO_2 particles were silylated; TiO_2 particles (4 g) were stirred to react uniformly with 3-(2-aminoethylpropyl) trimethylsilane (1%) for 60 min in 100 cm^3 dry benzene solution. Unreacted silane molecules were removed by washing with dry benzene, and then with methanol. Next, the silylated TiO_2 particles were reacted with Hp; in refluxing benzene solution, DCC was added as a hydrating agent, and a carboxyl group of Hp was bound through an amide-linkage with an amino group of the silylated TiO_2 particles under nitrogen atmosphere. The powder was filtered and the unreacted Hp was removed completely by washing with benzene and then with excess methanol until no fluorescence from free Hp molecules was observed in the filtered solution. Finally, the product (TiO_2 -Hp) was dispersed in water by sonication.

Structures of the unmodified TiO_2 , silylated TiO_2 and TiO_2 -Hp particles are shown in Fig. 1. The structure of TiO_2 -Hp powder was confirmed with a Fourier transform infrared reflection spectroscopy (FT-IR; Digilab Model FTS-15). The amount of TiO_2 in the TiO_2 -Hp particles suspension was measured as described previously⁴⁾. TiO_2 -Hp particles were sterilized

with an autoclave and finally diluted with a minimum essential medium (MEM, Gibco) solution to a desired concentration for cell experiments. Thus, MEM containing TiO_2 and TiO_2 -Hp particles were named as MEM- TiO_2 and MEM- TiO_2 -Hp, respectively. Surface charges of unmodified TiO_2 , silylated TiO_2 and TiO_2 -Hp particles dispersed in the cultured medium were measured on a zeta potential instrument (DELSA 440, COULTER, USA).

2.2 Cell line and transmission electron microscopy

HeLa cells were cultured in an MEM solution supplemented with 10% fetal calf serum in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C. For transmission electron microscopy studies, cells were cultured in an MEM- TiO_2 -Hp or in a MEM- TiO_2 (5 ml, 100 μg TiO_2 cm^{-3}) solution for 2 min, 3 h or 24 h. Then, the cells were collected and immediately prefixed in 2.5% glutaraldehyde solution buffered with 0.05 M sodium phosphate, pH 7.4, containing

Fig. 1 Schematic illustration of TiO_2 , silylated TiO_2 and TiO_2 -Hp particles.

0.05 M sucrose. Cells were postfixed in 1% osmium tetroxide using the phosphate buffer for 1 h at ice-both temperature. The cells were rinsed with H₂O for 10 min, then dehydrated using sequential ethanol and acetone washes. The cells were embedded in an epoxy resin (EPON 812) mixture, then sectioned with a microtome. The section was poststained with uranyl acetate and lead citrate. Finally, distribution of the TiO₂-Hp or TiO₂ particles in the cells was observed on a Hitachi H-7000 (75KV) TEM.

2.3 Fluorescence of intracellular TiO₂-Hp particles

Fluorescence microscope measurements were done by the similar procedure reported by Chan *et al.* with minor modification⁸⁾. About 10⁵ HeLa cells were cultured on a micro (12 mm) round cover glass in MEM-TiO₂-Hp (100 µg TiO₂ cm⁻³). The exposure times were also chosen as 2 min, 3 h and 24 h. After removal of the MEM-TiO₂-Hp solution, the cells were washed twice with phosphate buffered saline (PBS) solution, then covered with a cover glass. Finally, the cells were observed with a fluorescence microscope (BH2-RFL + Olympus, Japan). A

violet excitation unit ($\lambda_{\text{max}}=405$ nm) and an optical glass filter (407 K) were used to observe the red fluorescence from TiO₂-Hp. Photographs were obtained on a 400 ASA kodak film. In some experiments, MEM-TiO₂-Hp was replaced by Hp containing MEM for comparing the fluorescence of TiO₂-Hp with that of free Hp.

3 RESULTS AND DISCUSSION

3.1 Characterization of TiO₂-Hp

Figure 2 shows the FT-IR spectra of the unmodified TiO₂ (curve 2a), silylated TiO₂ (curve 2b) and TiO₂-Hp particles (curve 2c). In the case of the unmodified TiO₂ particles, the strong broad OH band around 3300 cm⁻¹ and adsorbed water band at 1630 cm⁻¹ were observed (curve 2a). After the TiO₂ particles were silylated, the intensity of the OH band at 3300 cm⁻¹ was reduced, and new bands at 2926 cm⁻¹ and 2870 cm⁻¹ caused by C-H of -CH₂- were observed. Moreover, new bands originated from Ti-O-Si and C-N (of -CH₂-NH₂) appeared at 1350 cm⁻¹ and 1148 cm⁻¹, respectively (curve 2b). These new bands were not observed in the unmodified TiO₂ particles, indicating that 3-(2-amino-ethylpropyl)

Fig. 2 FT-IR spectra of (2a) unmodified TiO₂ particles, (2b) silylated TiO₂ particles, (2c) TiO₂-Hp particles.

- trimethoxysilane was actually bound to the TiO_2 particles.

When Hp was linked to the silylated TiO_2 particles, a new sharp band of $\text{C}=\text{O}$ appeared at 1720 cm^{-1} . Moreover, $\text{N}-\text{H}$ band of hematoporphyrin ring was observed at 1650 cm^{-1} (curve 2c). It was also observed that the FT-IR spectrum of TiO_2 -Hp particles was different from that of the TiO_2 particles mixed mechanically with Hp; the band of $-\text{COOH}$ at 1735 cm^{-1} appeared in the mixed spectrum was shifted to 1720 cm^{-1} with TiO_2 -Hp particles, because Hp has two free $-\text{COOH}$ groups, but those $-\text{COOH}$ groups were bound with $-\text{NH}_2$ groups of silylated TiO_2 particles, forming amide linkage ($-\text{NHCO}-$) in TiO_2 -Hp particles as illustrated in Fig. 1. Therefore we can conclude that Hp is actually bound to the silylated TiO_2 particles.

It is well known that TiO_2 (anatase, P-25) particles have "fully hydroxylated surfaces". Munuera *et al.* reported that following three forms of adsorbed water exist on the surface of TiO_2 particles⁹⁾:

(1) as a form of loosely bonded water with a band at 1610 cm^{-1} ; this band is completely removed by heating it above 200°C

(2) as a form of hydroxyl groups of acidic character with a band at $3680\text{--}3580\text{ cm}^{-1}$.

(3) as a form of hydroxyl groups of basic character with a band at 3730 cm^{-1} .

The hydroxyl groups of acidic character were reported to react fairly easily with alcohols to form esters¹⁰⁾. In the present study, such acidic hydroxyl groups may be used predominantly in the modification reaction.

The quantity of Hp on TiO_2 particles was estimated to be about 4.2×10^{19} Hp/1g TiO_2 in TiO_2 -Hp particle system, corresponding to about 3×10^3 Hp on one TiO_2 particle. This quantity was determined by the following procedure. The amide linkage of TiO_2 -Hp was first broken by an alkali hydrolysis reaction (in 0.5 M NaOH solution). Then the TiO_2 particles were filtered and the concentration of Hp in the filtrate was determined by measuring its absorption spectrum. The quantity of OH groups on the TiO_2

particles used was reported to be about 1.4×10^{20} OH/1g TiO_2 particles¹⁰⁾, which is three times larger than that of Hp chemically modified on TiO_2 . In other words, about 30% of OH groups on the TiO_2 surface were combined with Hp in the present system. This value is in good agreement with the report of Andrew *et al.* for TiO_2 modified by cobalt(II)tetrakisulfophthalocyanine¹¹⁾.

It is known that surface charge of particles was changed by chemical modification¹²⁾. We measured the zeta potentials of unmodified TiO_2 and TiO_2 -Hp in MEM solution. The results are shown in Fig. 3, -27 mV for the unmodified TiO_2 and -40 mV for the TiO_2 -Hp particles.

Fig. 3. Zeta potentials of (3a) MEM- TiO_2 , (3b) MEM- TiO_2 -Hp.

3.2 Efficiency of phagocytosis of TiO_2 particles and TiO_2 -Hp particles in cultured cells.

In order to compare the efficiency of phagocytosis of unmodified TiO_2 with that of TiO_2 -Hp particles, the cells were treated with MEM- TiO_2 or MEM- TiO_2 -Hp for various incubation time, and then they were observed with the TEM. The TEM observations were performed with the following six samples. Sample 1, 2 and 3 were the cells treated with MEM- TiO_2 -Hp for 2 min, 3 h and 24 h, respectively. Similarly, sample 4, 5 and 6 were the cells treated with MEM- TiO_2 for 2 min, 3 h and 24 h, respectively. When the

treatment time was only 2 min (sample 1 and sample 4), neither TiO_2 -Hp nor TiO_2 particles were detected in the cells (Fig. 4a and Fig. 4b), meaning that these particles have not phagocytized by the cells yet. After 3 h of treatment, for sample 2, a fairly amount of TiO_2 -Hp powders were detected in the cytoplasm (Fig. 4c). After 24 h of treatment, for sample 3, the quantity of phagocytized TiO_2 -Hp was increased, as seen in Fig. 4d. The TiO_2 -Hp particles aggregated in the cytoplasm as shown in Fig. 4e and the size of the aggregated particles after 24 h treatment was larger than that after 3 h treatment. On the other hand, the unmodified TiO_2 particles were not phagocytized into the cytoplasm, but were only adsorbed on the cell membrane after 3 h of treatment, for sample 5, as shown in Fig. 4f. Such unmodified TiO_2 particles were observed in the cytoplasm only with sample

(4a) MEM- TiO_2 -Hp, Treatment time: 2 min. TiO_2 -Hp particles were not phagocytized by the cells.

(4b) MEM- TiO_2 , Treatment time: 2 min. TiO_2 particles were not phagocytized by the cells.

6 (after 24 h of treatment), as seen in Fig. 4g. These results clearly indicate that the TiO_2 particles modified with Hp are easier to be phagocytized by the cells than the unmodified TiO_2 particles. This conclusion was also supported by our preliminary experiments that the ability in "photokilling" of the cells following 24 h of treatment with TiO_2 -Hp was larger than that with unmodified TiO_2 particles.

The other interesting observation is that both the TiO_2 and TiO_2 -Hp particles existed only in the cytoplasm, but not in the nucleus. This phenomenon is similar to that of phagocytized crystalline Ni_3S_2 particles reported by Costa *et al.*¹³⁾ However, the cytotoxicity of TiO_2 is quite different from that of Ni_3S_2 . We reported recently that cells were all viable even when $100 \mu\text{g cm}^{-3}$ of TiO_2 particles was added to the cells for 24 h without light irradiation⁴⁾. On the other hand, cytotoxicity of Ni_3S_2 was reported to be large; its LD_{50} (i.e., dose of Ni_3S_2 particles needed to inactivate 50% of the cells) was only $2 \mu\text{g cm}^{-3}$. Its large cytotoxicity is explained as follows. Although the phagocytized Ni_3S_2 particles do not enter the nucleus, they dissolve rapidly in the cytoplasm. This dissolution allows entry of ionic nickel into the nucleus, resulting in the cell death. On the other hand, TiO_2 is so stable that cytotoxicity is negligibly small.

The phagocytosis of TiO_2 -Hp was also monitored on a fluorescence microscope by exciting TiO_2 -Hp particles with 405 nm light. The

(4c) MEM- TiO_2 -Hp, Treatment time: 3h. Some TiO_2 particles were adsorbed on the cell membrane, and some of them were phagocytized into cytoplasm.

(4d) MEM-TiO₂-Hp, Treatment time: 24 h. A fairly amount of TiO₂ particle was phagocytized into cytoplasm.

(4e) MEM-TiO₂-Hp, Treatment time: 24 h. Large size of TiO₂ particles (aggregated) were found in the cytoplasm.

(4f) MEM-TiO₂, Treatment time: 3h. TiO₂ particles were adsorbed on the cell membrane, but were not phagocytized into cytoplasm.

(4g) MEM-TiO₂, Treatment time: 24 h. TiO₂ particles were phagocytized into cytoplasm.

Fig. 4 TEM picture of HeLa cells after MEM-TiO₂ (TiO₂ = 100 $\mu\text{g cm}^{-3}$) solution was added to the cells.

experimental procedure was almost the same as that of TEM observation. When MEM-TiO₂-Hp (100 $\mu\text{g cm}^{-3}$) was added to the cells for 2 min, no fluorescence of TiO₂-Hp was observed, indicating that the TiO₂-Hp particles were not phagocytized by the cells yet. After 3 h of treatment, fluorescence from TiO₂-Hp was clearly observed around the nucleus of the cells (Fig. 5a)., It means that some TiO₂-Hp particles were phagocytized by cells following 3 h of treatment. After 24 h treatment, stronger fluorescence from TiO₂-Hp showed particle-shape around the nucleus of the cells (Fig. 5b), showing that more TiO₂-Hp particles were phagocytized after longer treatment time. Thus, these results agree well with the TEM's observation.

From fluorescence observation, one can find

that TiO₂-Hp particles showed particle-shape fluorescence. However, when MEM-TiO₂-Hp was replaced by Hp containing MEM, fluorescence came uniformly from the cytoplasm due to a large affinity of Hp for the cells¹⁴⁾. This uniform fluorescence of Hp was obviously different from that of TiO₂-Hp.

The difference in uptake between TiO₂ and TiO₂-Hp may be mainly due to the larger affinity of Hp for HeLa cells than that of TiO₂ powder itself. Actually, it is said that Hp moleculars are easily uptaken by cells and localized selectively in tumor^{15, 16)}, therefore they are used in cancer photodynamic therapy. Since phagocytosis of particles involves an interaction between the particles surface and the cellular membrane, characters of particle surface may also play an

5a

(5a) Treatment time: 3 h. Fluorescence of $\text{TiO}_2\text{-Hp}$ shows particle-shape around the nucleus and it does not come from the nucleus of the cells.

5b

(5b) Treatment time: 24 h. Stronger fluorescence of $\text{TiO}_2\text{-Hp}$ shows particle-shape around the nucleus of the cells.

Fig. 5 Fluorescence photograph of $\text{TiO}_2\text{-Hp}$ particles in the cells after $\text{TiO}_2\text{-Hp}$ ($\text{TiO}_2=100 \mu\text{g cm}^{-3}$) solution was added to cells.

important role in phagocytosis. In this sense, the shift of TiO_2 surface charge to negative direction by the modification with Hp may be the other reason for the more efficient phagocytosis. Actually, in the study of "cell-particle interaction", Abbracchio *et al.*¹⁷⁾ reported that crystalline NiS negatively charged is phagocytized by cells, whereas amorphous NiS positively charged is not phagocytized. Based on the video-time-lapse microscope observation, Evans *et al.*¹⁸⁾ also suggested that the binding and uptakes occur at ruffling areas of the cell membrane, where the membrane charge is

altered, and only the negative particles are phagocytized. Similar mechanism could explain our result on the present TiO_2 system.

The particle transport in the cell membrane is one of the current topics in particle-cell interaction. For instance, Sheetz and collaborators used gold particles (40 nm) to trace the transport of particle in the cell membrane¹⁹⁾. Peter *et al.*²⁰⁾ applied magnetic Fe_2O_3 particles as a probe for intracellular particles movement, since magnetic Fe_2O_3 particles can be controlled by an external magnetic field. From this viewpoint, $\text{TiO}_2\text{-Hp}$ is also a good probe to monitor the particle transport in the cell by fluorescence microscope.

Human epidemiological and laboratory studies using experimental animals have shown that a number of particles (SiO_2 , NiS , Ni_3S_2) are carcinogenic. TiO_2 particles using in this study, however, showed no toxicity to the cultured cells, but showed a large effect of "photokilling" of cells under UV irradiation⁴⁾. We believe that TiO_2 powders injected into the experimental animals can destroy the tumor tissue under irradiation. Now a study in this line is on the way.

One of the authors (R. Cai) would like to express his gratitude to President H. Komiya of Bionics Co. for his financial support.

REFERENCES

- 1) B.K. Bernard, M.R. Osheroff, A. Hofman and J.H. Mennear, *J. Toxicol. Environ. Health*, **28**, 415 (1989).
- 2) National Cancer Institute. NCI carcinogenesis Technical Report No. 97 (1979).
- 3) F. Bischoff and G. Bryson, *Res. Commun. Chem. Pathol. Pharmacol.*, **38**, 279 (1982).
- 4) R.X. Cai, K. Hashimoto, K. Itoh, Y. Kubota and A. Fujishima, *Bull. Chem. Soc. Jpn.*, **64**, 1268 (1991).
- 5) M. Fujihira, Y. Satoh and T. Osa, *Nature*, **293**, 206 (1981).
- 6) T. Inoue, A. Fujishima, S. Konishi and K. Honda, *Nature*, **277**, 637 (1979).
- 7) A. Fujishima, J. Otsuki, T. Yamashita and S. Hayakawa, *Photomed. Photobiol.*, **8**, 45 (1986).
- 8) W.S. Chan, R. Sversen, D. Philips and I.R. Hart, *Br.J. Cancer*, **53**, 225 (1986).
- 9) G. Munuera, V. Rives-Arnau and A. Saucedo, *J.C.S. Faraday Trans I*, **75**, 736 (1979).

- 10) H.P. Boehm, *Discuss. Faraday. Soc.*, **52**, 264 (1971).
 - 11) P. H. Andrew, D.W. Bahnemann and M.R. Hoffmann, *J. Phys. Chem.*, **91**, 2109 (1987).
 - 12) K. Esumi and K. Meguro, *Bull. Chem. Soc. Jpn.*, **56**, 331 (1983).
 - 13) M. Costa and H.H. Mollenhauer, *Cancer Res.*, **40**, 2688 (1980).
 - 14) M. Berns, A. Dahlman, F. Johnson, R. Burns, D. Sperling, M. Guiltinan, A. Siencns, R. Walter, W. Wright, M. Wilson and A. Wile, *Cancer. Res.*, **42**, 2325 (1982).
 - 15) T. Dougherty, *Photochem. Photobiol.*, **45**, 879 (1987).
 - 16) D. Kessel, *Photochem. Photobiol.*, **39**, 851 (1984).
 - 17) M.P. Abbracchio, J. Simmons-Hansen and M. Costa, *Toxicol. Environ. Health*, **9**, 663, (1982).
 - 18) R.M. Evans, J. Peter, A. Davies and M. Costa, *Cancer Res.*, **42**, 2729 (1982).
 - 19) M.P. Sheetz, S. Turmey, H. Qian and E.L. Elson, *Nature*, **340**, 284 (1989).
 - 20) G. Peter, D.B. Joseph, B.B. Steven and A.V. Peter, *Nature*, **302**, 336 (1983).
-