Photokilling of Malignant Cells with Ultrafine TiO_2 Powder

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Photo-irradiated TiO_2 particles drive various chemical reactions due to their strong oxidation and reduction ability. This effect is applied in order to kill malignant HeLa cells with TiO_2 particles. During 10-min UV irradiation periods, HeLa cells were completely killed in the presence of TiO_2 (100 $\mu g\,cm^{-3}$). However, without irradiation these particles showed little cytotoxicity up to 360 $\mu g\,cm^{-3}$. It is concluded that the cells were killed by hydroxyl ($OH\cdot$) and perhydroxyl ($HO_2\cdot$) radicals produced from water by the irradiated TiO_2 particles, and also that the cells were directly oxidized by photogenerated holes in TiO_2. The distribution of TiO_2 powders in the cells was observed using a transmission electron microscope. The TiO_2 powder existed not only on the cell membrane, but also in the cytoplasm. Thus, the cells were damaged by photoexcited TiO_2 not only from the cell membrane, but also from the inside of cytoplasm. Finally, the application of this malignant cell photokilling effect to photodynamic therapy is discussed.

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 and causing various chemical reactions. Photochemical reactions using TiO_2 powders are therefore being studied for solar energy conversion applications,[1-2] and photo-organic synthesis research,[3-5] Few studies, however, have investigated biochemical reactions using the semiconductor photoeffect. Matsunaga et al. reported the photocatalytic sterilization using TiO_2 powder. In their system, microbial (*Escherichia coli*) cells were killed by photocatalytic reaction.[6] The present study proposes applying heterogeneous photocatalysis with TiO_2 dispersion to a biochemical reaction in order to "photo-kill" cancer cells.

It was previously reported that HeLa cells could be photoelectrochemically killed on a TiO_2 electrode.[7] In this system, HeLa cells were cultured on TiO_2 electrode surface, and the electrode was anodically polarized under UV-irradiation. Photoexcited TiO_2 anode has a strong oxidation power, so that the cells on the electrode are damaged from the cell membrane.

Instead of using an electrode, the present study uses small TiO_2 particles which function as a "short-circuit," photoelectrochemical cell. In this "particulate system," both photogenerated electrons and holes reach the surface, thus oxidation and reduction are induced on the surface. Additionally these particulate suspensions yield a large interfacial surface area in small volumes. The ability of a photochemical reaction using particulate TiO_2 becomes higher when the size of particles becomes smaller,[8] allowing the particles to be easily incorporated into the cells. Therefore this method is easier to apply than the electrode method. The effects of small photoexcited TiO_2 particles on cells is an attractive investigation area because it is expected that the small particles are incorporated inside the cell, and the particulate reaction with the cellular components can more effectively proceed as compared to the electrode method.

In the present study, the cytotoxicity of ultrafine TiO_2 powder and the effect of photoexcited TiO_2 particles on malignant cells were investigated. Distribution of TiO_2 particles in the cells was also examined with a transmission electron microscope (TEM), and a cyclic voltammogram technique was applied to get electrochemical information. The possibility to apply this photocatalytic reaction to photodynamic therapy is also discussed.

Materials and Methods

TiO_2 Powder: TiO_2 particles (anatase, p-25; Nippon-Aerosil Co., Tokyo, Japan) with an average size of 300 Å in diameter were used. The particles were ultra-sonically dispersed in water, then sterilized using autoclave. During sterilization the TiO_2 particles aggregated and could not be sonically dispersed, thus they were removed by centrifugation, and the small aggregates were then collected. The average size of TiO_2 aggregates in the suspension, observed by a scannig electron microscope (SEM) [JSM-840, JEOL., Tokyo, Japan], depended on the centrifugal acceleration, with the TiO_2 suspension quantity being measured with combustion analysis. The optimum acceleration was chosen to be 1600×g in the present experiment. This will be discussed later. A minimum essential medium (MEM, Gibco) solution (5×MEM) and 10% fetal bovine serum were added to the ultrafine TiO_2 aqueous suspension to prepare a MEM solution containing TiO_2 (MEM-TiO_2 solution), which was used to investigate both the photokilling ability (with irradiation) and the cytotoxicity (no irradiation) of HeLa cells.

Cell Lines and Antitumor Activity of the TiO_2 Powder: The 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.

The antitumor activity of the ultrafine TiO_2 aggregates

was evaluated with a colony forming assay (plating efficiency: 80—85%). The cells were plated in 60 mm petri dishes (Becton-Dickinson Co.), and after incubated for 24 h at 37 °C in 5% CO_2 allow cell attachment, the old MEM solution was replaced with an MEM-TiO_2 solution. The cells were then incubated for 24 h, the solution was removed, and the cells were washed twice with a Hanks' balanced salt solution (Gibco). A TiO_2-free medium was finally added to the cells and the prepared cells were irradiated with a 500-W high pressure Hg lamp (USHIO Co., Tokyo, Japan) at room temperature. For the visible light experiments a sharp cut-off filter (L-44, Kenko Co., Tokyo, Japan) was used to get a light wavelength greater than 440 nm. For the UV light experiments a UV pass filter (UV-D2, TOSHIBA Co., Tokyo, Japan) was used to get a light wavelength between 300—400 nm. After culturing again for 10 days, the colonies were fixed with 70% methanol, stained with a 5% Giemsa solution, and then counted. In cytotoxicity experiment, cultures without drug in dark were used as controls. In irradiation experiment, cultures exposured to the various drugs as described above but not exposured to light were used as controls in irradiation experiments. Each experiment was done using three dishes at the same time and under the same conditions. Then those three data were averaged. Moreover, those experiments were repeated 3 to 4 times. The error bars in the figures represent the maximum and minimum value at each experiment. During the irradiation a water filter was used to remove infrared radiation, and light doses were measured by a photodiode.

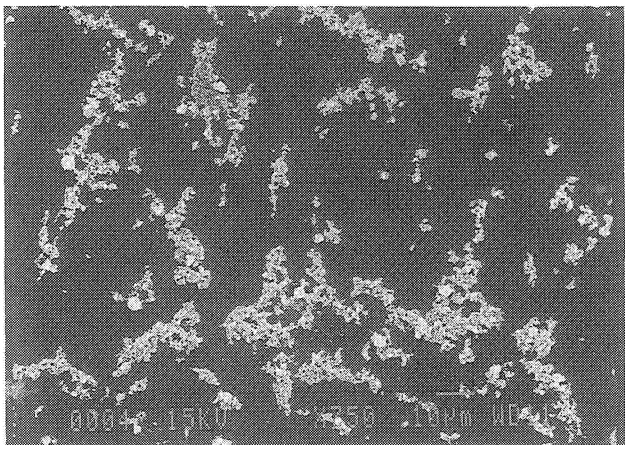
Transmission Electron Microscope Measurement: HeLa cells were incubated in an MEM-TiO_2 (TiO_2=100 $\mu g\,cm^{-3}$) solution for 24 h, and then were collected and immediately fixed using a 2.5% glutaraldehyde solution at 4 °C. In some experiments, TiO_2 aggregates whose size was smaller than 0.22 μm were used. This small aggregates were obtained by filterating the centrifugated TiO_2 solution with a 0.22 μm milipore filter. After 2 h the cells were fixed again with a 2% osmium tetraoxide, then dehydrated by gradually increasing the concentration of alcohol, and embedded in synthetic resin (EPON 812). Semi-thin (2 μm) sections of the cell were stained with methylene blue, which were then made into ultrathin sections (0.05—0.1 μm) by using a Reihert ultramicrotome and a diamond knife, and then double stained with uranyl acetate and lead(II) citrate. Finally, distribution of the ultrafine TiO_2 particles in the cells was observed with a Hitachi H-7000 (75 kV) TEM.

Electrochemical Measurements: Cyclic voltammograms were taken using a slightly modified three-electrode system.[9] A graphite electrode, polished before each run (0.4 cm^2 surface area), a counter electrode (platinum wire), and a reference electrode (saturated calomel electrode: SCE) were fitted into the top of a glass vessel (25 cm^3 capacity). A sufficient number of HeLa cells were first dropped on the membrane filter while applying a slight suction, and were retained on the membrane filter surface. This filter was attached to the surface of the graphite electrode, which was then inserted into phosphate buffered saline [PBS] (pH 7.0, 15 cm^3) to get the cyclic voltammograms, obtained by using a potentiostat (Toho Technical Research Co., Tokyo, Japan; Model UFB-4), function generator (Hokuto Denko Co., Tokyo, Japan; Model HB-III), and X-Y recorder (Riken Denshi Co., Tokyo, Japan; Model F-5c).

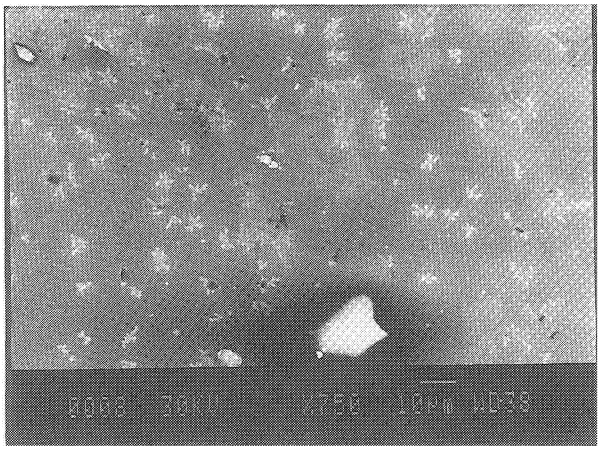
Results and Discussion

Cytotoxicity of TiO_2: Investigation of the cytotoxicity of a drug, without irradiation, is important before it can be used as a photodrug, i.e., antitumor drugs used in PDT are required to be almost nontoxic, and yet have a high photokilling ability under irradiation.

The initial TiO_2 particles had an average size of 300 Å in diameter, and since hydroxyl groups (-OH) exist at the surface of the particles,[10] the hydrogen bond between the hydroxyl groups is formed in the aqueous solution, resulting in aggregation of the particles. Figure la shows an SEM image of the particles in the suspension. The average size of the aggregated TiO_2 was much larger (20 μm) than that of the initial ones. It was found that such large aggregated TiO_2 particles showed a high cytotoxicity to the cells. This is probably because they deposit and covered the



(a)



(b)

Fig. 1. SEM pictures of TiO_2 particles
(a) without centrifugation, (b) after centrifugation at 1600×g.

cells. However, the large aggregated particles can be removed by centrifugation and small aggregates in the suspension can be obtained. The size of TiO_2 aggregates depended on the centrifugal acceleration. Smaller TiO_2 aggregated can be obtained using a higher acceleration. However, if the acceleration was too high, the concentration of TiO_2 in the suspension decreased, therefore the optimum acceleration was chosen to be 1600×g, with the size of the TiO_2 particles being in the range of 0.03—10 μm (Fig 1b), much smaller than the size of HeLa cells (50 μm).

When HeLa cells were incubated in an MEM-TiO_2 for 24 h (without irradiation), the surviving fraction of the cells with respect to the amount of TiO_2 powder is shown in Fig. 2. The surviving fraction was greater than 90% when the concentration of MEM-TiO_2 reached 360 $\mu g\,cm^{-3}$, and it is subsequently concluded that the small TiO_2 particles showed little cytotoxicity without irradiation.

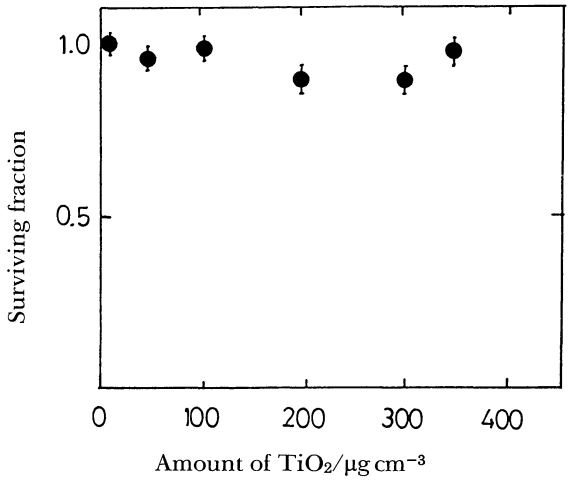


Fig. 2. After HeLa cells were incubated with an MEM-TiO_2 solution for 24 h without irradiation, the surviving fraction of HeLa cells is shown as a function of the amount of ultrafine TiO_2 particles.

Photokilling of Cells Using TiO_2 Powders: The irradiation effects when TiO_2 is not used are shown in Fig. 3, with the surviving fraction of HeLa cells given as a function of the unfiltered light irradiation time. The 500-W Hg lamp emitted a relatively strong UV light, thus a significant number of cells were killed after a 25 min exposure, whereas after a 10 min exposure, more than 90% of the cells remained alive.

When TiO_2 was added, the HeLa cells were killed at a much higher rate as shown in Fig. 3. Notice when the amount of TiO_2 was 12 $\mu g\,cm^{-3}$, 20% of the cells were killed after 5 min of irradiation, and also that when the amount of TiO_2 was 95 $\mu g\,cm^{-3}$ and 120 $\mu g\,cm^{-3}$, 75% and 90% of the cells were respectively killed. The ultrafine TiO_2 particles showed little cytoxicity at 100 $\mu g\,cm^{-3}$, Therefore, it is concluded that under irradiation the ultrafine TiO_2 particles can actively kill the cells.

When the cultured cells are exposed to the white light from the 500-W Hg lamp, the semiconductor TiO_2 absorbs only the light whose energy is larger than its band gap (the TiO_2 band gap is 3 eV, corresponding to a wavelength of 410 nm). In order to prove whether the cell death was caused by a photo process or a thermal process, the following experiments were done. First the temperature increse of MEM-TiO_2 caused by the light irradiation was measured. The result is shown in Fig.4. In the beginning, it was 27 °C, and was raised up to about 36 °C after 30 min irradiation. However, that temperature was lower than that at culture environment (37 °C). Therefore it is reasonable to expect that such a temperature increase does not affect on the cell. Actually it was reported that the thermal effect is observed at the temperature is higher than 41 °C.[11] Next, a sharp cut-off filter (L-44) was inserted between the Hg lamp and the cells, allowing only light with wavelength larger than 440 nm to irradiate. Figure 5 shows that even after a 25 min irradiation more than 90% of the

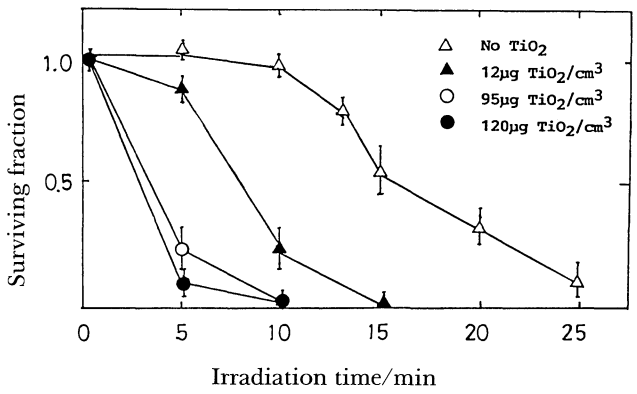


Fig. 3. Surviving fraction of HeLa cells as a function of unfiltered irradiation light in the presence of different concentrations of MEM-TiO_2 solutions.

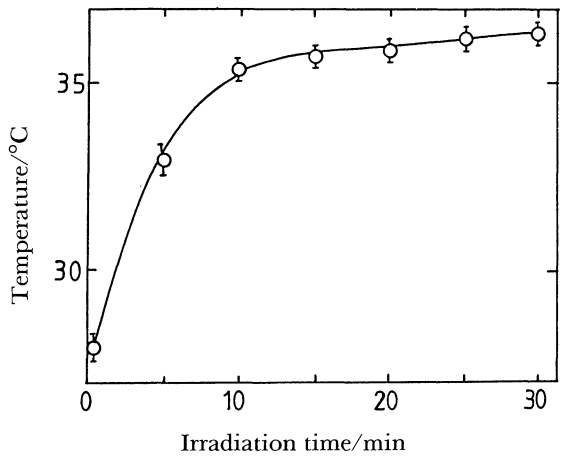


Fig. 4. Temperature increase of MEM as a function of irradiation (white light) time.

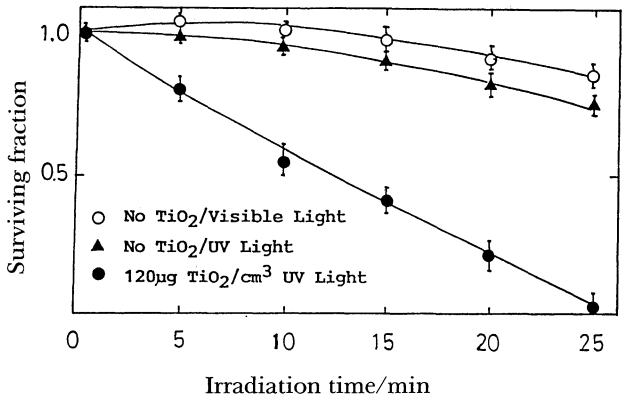


Fig. 5. Surviving fraction of HeLa cells as a function of visible light and UV irradiation with no TiO_2, and UV light irradiation with TiO_2. An MEM-TiO_2 solution (120 $\mu g\,cm^{-3}$) was added to the HeLa cells for 24 h before the UV irradiation.

cells were alive. It indicated that light with wavelength larger than 440 nm do not cause the photokilling effect. Finally, a UV-D2 filter was then used so that only light with wavelength between 300—400 nm irradiated. After a 25 min irradiation (TiO_2=120 $\mu g\,cm^{-3}$) all cells were completely killed, whereas in the absence of TiO_2 more than 85% of the cells remained alive. These results clearly show that the cell death in the presence of TiO_2 is caused by a photoeffect, not a thermal effect.

Distribution of TiO_2 Powder in the Cell: After the cells were incubated with MEM-TiO_2 (TiO_2=100 $\mu g\,cm^{-3}$) for 24 h, the TiO_2 distribution was observed with the TEM as shown in Fig. 6. TiO_2 particles were found on the cell membrane and in the cytoplasm, but not in the nuclei of the cells.

Cells can incorporate small particles by the process of phagocytosis, thus it can be resonably assumed that the same process takes place when the MEM-TiO_2 solution was added to the cells.

Moreover, it was found that the TiO_2 in the cytoplasm was gathered forming larger aggregates. This was concluded by the following experiment. That is, when TiO_2 aggregates whose size was smaller than 0.22 μm were incorporated, much larger aggregates (1.5 μm) were observed in the cytoplasm as shown in Fig. 7.

The observation that TiO_2 exist not only on the cell membrane, but also in the cytoplasm is very important to consider the reason why the present system effectively kill the cells. The organs in the cell were also damaged by the TiO_2. This point is quite different from the mechanism of TiO_2 electrode system. In the latter system, only the cell membrane is damaged by photoexcited TiO_2.

Reaction Mechanism: Two different photochemical processes are proposed which kill the HeLa cells in the present system. The first is the direct reaction between photoexcited TiO_2 and the cells. It is well known that the photoexcited TiO_2 is a strong oxi-

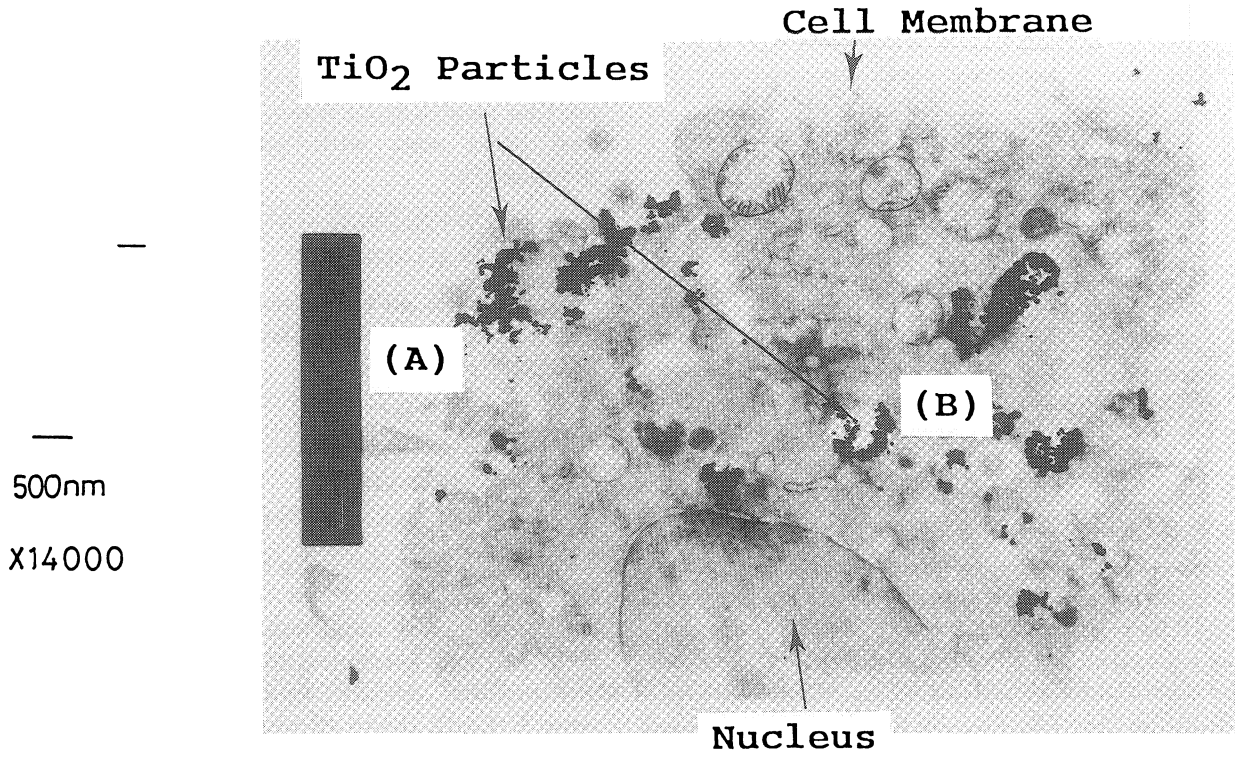


Fig. 6. TEM picture of HeLa cells after the TiO_2-MEM (TiO_2=100 $\mu g\,cm^{-3}$) solution was added to the cells for 24 h. TiO_2 aggregates were found on the cell membrane (A), and in the cytoplasm (B).

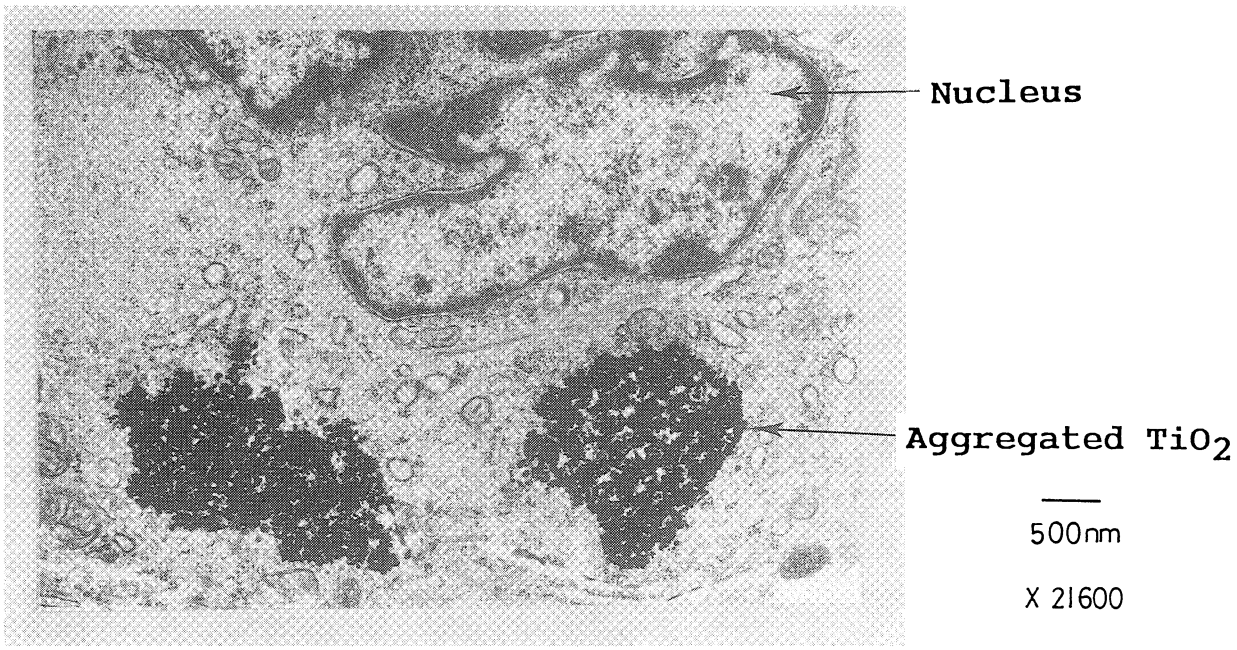


Fig. 7. TEM picture of HeLa cells after the ultrafine TiO_2 (0.03—0.22 μm) was added to the cells for 24 h. The TiO_2 particles was obtained using 0.22 μm milipore filter.

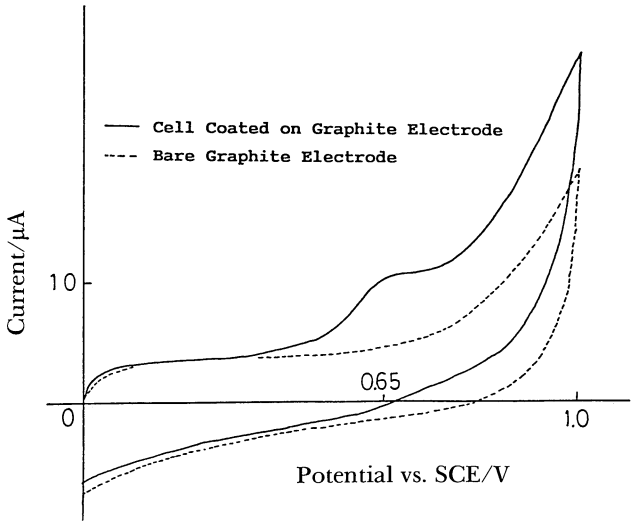


Fig. 8. Cyclic voltammogram of (——) a graphite electrode coated with HeLa cells and (----) a bare graphite electrode, in a 0.1 M phosphate buffer (pH 7.0). 1 M=1 mol dm^{-3}.

dizer.[12] Therefore it is expected that the cells are directly oxidized by the holes formed in the valence band. In order to determine the oxidation potential of the HeLa cells, we measured cyclic voltammograms of the cells adsorbed on a graphite working electrode in the potential range of 0 to 1.0 V vs. SCE in PBS (pH 7.0) solution (See Fig. 8). An anodic peak appeared at 0.65 V vs. SCE in the first positive forward direction scan, with peak intensity increasing as the number of HeLa cells increases on the electrode. However, no corresponding reduction peak was observed during the negative backwards direction scan, indicating that the HeLa cell oxidation process occurred in the potential range of 0.65 V vs. SCE. Since the energy level of valence band edge of TiO_2 is reported to be 2.4 V vs. SCE at pH 7,[12] it is reasonable to assume that the photogenerated holes in TiO_2 can oxidize the cells.

The other proposed process is the indirect reaction between photoexcited TiO_2 and the cells. Hydroxyl radicals (OH·) and/or perhydroxy radicals (HO_2·) are first created from water. When the photogenerated holes react with water, OH· radicals are produced.[13]

$$(TiO_2) + h\nu(>3\ eV) \rightarrow h^+ + e^- \quad (1)$$

$$H_2O + h^+ \rightarrow OH\cdot + H^+ \quad (2)$$

Additionally, in the presence of oxygen in water, it is reported that both HO_2· and OH· radicals are formed as follows.[13]

$$H^+ + e^- \rightarrow H\cdot \quad (3)$$

$$O_2 + e^- \rightarrow O_2^{-\cdot} \xrightarrow{H\cdot} HO_2^- \quad (4)$$

$$HO_2^- + h^+ \rightarrow HO_2\cdot \quad (5)$$

$$2HO_2\cdot \rightarrow O_2 + H_2O_2 \xrightarrow{O_2^{-\cdot}} OH\cdot + OH^- + O_2 \quad (6)$$

Those radicals may attack the cell membrane and cellular components, and oxidation-reduction substances which are important for producing ATP in a HeLa cell such as reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide(NADH), coenzyme A (CoA), flavin adenine dinucleotide ($FADH_2$), may react with these radicals. The cell will die if these substrates are either depleted or exhausted. Preliminary experiments confirmed that in the presence

of cystein and catalase, respectively scavengers of OH· and H_2O_2, the surviving fraction significantly recovered.

Application of Photocatalytic Reaction of TiO_2 to Photodynamic Therapy: Research on light absorbing anti-cancer drugs is studied in biological systems, with these drugs being exposed to UV or visible light.[14-16] Extensive clinical trials have been performed using HpD as a PDT sensitizer because it is selectively localized in tumors. Further improvement of PDT can be envisaged through the use of more efficient photosensitizers.

In the HpD system two different mechanisms have been found that cause cell damage i.e., the sensitizer molecules excited in the lowest triplet state react directly with biological substrates,[17-18] and the photogenerated triplet state of the sensitizer transfers energy to oxygen to produce singlet molecular oxygen which damages cells by reacting with various biological substrates.[19-20] These two mechanisms are based on photochemical reactions, however, hyperthermal processes are also believed to photokill the tumor cells during irradiation.[21]

In the present system HeLa cells were definitely killed by a photochemical reaction and not by hypothermal effects. This large photokilling ability may be applied to PDT because the TiO_2 particles could be injected into the tumor, irradiated by UV light by laser fiber, thus destroying the tumor.

The UV light is strongly absorbed by patient's body, so the present system may not be useful for PDT of tumor formed in deep part of body. For a tumor formed near skin, such as skin tumor, however, this strong photoeffect may be useful. Compared to the conventional PDT of tumor using HpD, the present system has an advantage that cancer cell can be killed by a rather weak light intensity. We are now applying this method to animal experiment, i.e., bladder tumor of rat.

However, photoexcited TiO_2 has a very strong oxidation power and TiO_2 particles are incorporated not only by cancer cells but also by normal cells. Therefore, if UV light is irradiated uniformly, both the cancer cells and the normal cells are killed. This problem can be solved by focusing the light only on the tumor. Such a site selectivity is one of the superior characteristics of photodynamic therapy.

The other method to kill only the cancer cell is to bind TiO_2 particles to an HpD, causing them to be selectively localized in tumor, and thereby selectively killing the tumor during irradiation. In this case, the tumor formed not only near skin, but also near the surface of the organ, e.g. bladder, stomach, lung, can be killed by using strong photoeffect of TiO_2.

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