Anticancer Effect of Dye-sensitized TiO_2 Nanocrystals by Polychromatic Visible Light Irradiation

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TiO_2 nanocrystalline particles with monolayer adsorption of chlorin e6 trisodium salt (dye-TiO_2 particles) were administered into a cancer cell, murine thymic lymphoma (EL-4). Irradiation of the polychromatic light (550–750 nm) that excites chlorin e6 significantly damaged the EL-4 cells. A higher cell-killing effect was found for the dye-TiO_2 particle than for the system using chlorin e6 alone.

Photocatalytic activity of the oxide semiconductor, TiO_2, has been extensively applied to decomposition of organic species in environmental cleaning systems.[1] The origin of decomposition is generally accepted as the production of reactive radicals such as superoxide anion (O_2^-) by the conduction-band electron of the photoexcited TiO_2. This method has also been applied to medical treatment for cancer tissues. Cai et al. reported that photoexcited TiO_2 particles completely killed HeLa cells and T-24 human bladder cancer cells cultivated in vitro.[2,3] They verified that the reactive oxygen species generated by the photoexcited TiO_2 acts as a cytotoxin on the cell membrane and in the cytoplasm. The photocytotoxicity of TiO_2 is obtained by UV light irradiation with a wavelength region 300–400 nm that directly excites TiO_2. The penetration depth of UV light into human tissues is however limited to a few millimeters. Being largely absorbed by the skin, UV light cannot reach at the depth of the tissues[4] where cancer exists. Visible light of a wavelength range from 600 to 700 nm has high penetration ability.[5] In photodynamic therapy (PDT), use of visible light-sensitive dyes (sensitizers) such as hematoporphyrin derivatives has been extensively studied. Among sensitizers, chlorin e6, (Chl-e6) absorbing at 670 nm is an ideal dye. Ogura et al. showed the photocytotoxicity of conjugated chlorin and Chl-e6 in Hela cells and found high toxicity over nonconjugated dyes.[6,7] These backgrounds led us to assess photocytotoxicity of dye-sensitized TiO_2 particle as an anticancer agent for PDT.

Nanocrystalline TiO_2 was chosen for this purpose taking into consideration the penetration feasibility of the nanoparticle across the cellular wall. Dye sensitization of nanocrystalline TiO_2 is an established method for measuring photoelectrochemical effects. The positively charged surface of TiO_2 in polar solvents[8,9] accepts chemical adsorption of negatively charged dye molecules bearing carboxyl and hydroxy groups. For application to PDT, however, the dye-adsorbed TiO_2 particle (dye-TiO_2) should be prepared in an aqueous medium. No organic solvent may be introduced as a toxic contaminant with a clinical agent. Secondly, the dye–TiO_2 should be in the form of well-dispersed, nonaggregated particles to permit the cell penetration. Third, because of neutral pH desired for clinical treatment, dye molecule should be adsorbed to TiO_2 in the neutral water phase. To meet these conditions, we chose as a sensitizer chlorin e6 trisodium salt, which is water-soluble. The ability of Chl-e6 to sensitize TiO_2 has been photoelectrochemically verified by Amao et al.[10] Although conjugate materials of Chl-e6 have been examined in PDT,[6,7,11] no attempt has been ever made to use dye-sensitized nanoparticle in the field of medical treatments and PDT.

A neutral aqueous sol of nanocrystalline TiO_2 (anatase) with an average diameter of about 6 nm (TKS-203, TAYCA Co., Ltd.) was mixed with water and a dilute aqueous solution of Chl-e6 trisodium salt as a dye. Resultant 1.0 wt % TiO_2 particle aqueous dispersions that contain Chl-e6 at various concentrations were stirred for 2 h to yield opaque dispersions of Chl-e6-adsorbed TiO_2 nanocrystals.

The dye-sensitization effect of Chl-e6 absorbed on nanocrystalline TiO_2 (dye-TiO_2) was examined by fabricating a photoelectrochemical cell using iodide/triiodide system as a redox electrolyte.[12] Chl-e6-sensitized photocurrent occurs in a wavelength region from 470 to 700 nm, exhibiting two main bands at 500 and 670 nm, reflecting the optical absorption spectrum of Chl-e6 adsorbed on TiO_2. In an optimum condition of adsorption, quantum efficiency of sensitization was found to reach more than 60%. The result indicates that the visible light excitation of dye-TiO_2 causes efficient production of conduction-band electrons in the TiO_2.

To investigate the anticancer effect of the dye–TiO_2 particle, murine thymic lymphoma cells, EL-4 cells, cultivated in vitro were used. 400 μL of the dye–TiO_2 particle dispersion was administered into 5 mL of EL-4 cell dispersion (5×10^5 cells/mL) cultivated with RPMI 1641 medium, followed by incubation for 24 h at 37 °C. Fluorescence microscope observation for the incubated EL-4 cell showed that a part of the cell nuclei was actually invaded by Chl-e6 as visualized by week fluorescent image. After washed out, the EL-4 dispersion was irradiated for an hour by the polychromatic visible light (40 mW/cm^2). The light had a continuous spectrum with a wavelength range of 550 to 800 nm, including characteristic line spectra around 580, 630, and 670 nm. As a control, cell dispersions prepared with Chl-e6 alone (Chl-e6 solution without TiO_2) and TiO_2 alone (nonsensitized) were examined. The determination was repeated three times for each sample. Figure 1 exhibits the changes in anticancer effect of the photoexcited dye–TiO_2 particles for EL-4 cells with different dye concentrations. In Figure 1, the survival rate (mean ± SD) denotes the percentage of the number of survival cells in the treatment against the control defined as the number of survival cells incubated with the dispersion, but not exposed to the light; the smaller the survival rate is, the greater the anticancer effect is. Cell samples without light irradiation as a reference maintained nearly 100% survival rate, indicating no cell-killing effect in the dark.

The survival rate in the presence of dye–TiO_2 gradually decreased with increasing dye concentration to 6% at a low

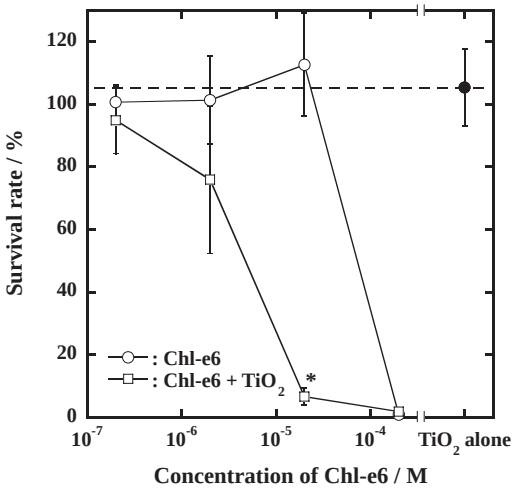

Figure 1. Changes in survival rate of dye–TiO_2 particle dispersion and aqueous Chl-e6 solution against concentration of Chl-e6. The dashed line denotes the rate of the control TiO_2 dispersion. The value of rate is mean ± SD ($n = 3$). ∗: $p < 0.05$ compared by Student's *t*-test with the survival rate in the administration of Chl-e6 alone at the same concentration.

concentration of 2×10^{-5} mol L^{-1} and reached about 0% at 2×10^{-4} mol L^{-1}. In contrast, the survival rate in the TiO_2-free Chl-e6 system remains 100% up to 2×10^{-5} mol L^{-1} and was suppressed to 0% at a high concentration of 2×10^{-4} mol L^{-1}, where Chl-e6 accumulated in the cells had the cell-killing effect. The survival rate of 1.0 wt % TiO_2 dispersion, as the dye-free control, was proved to be 105.4 ± 12.2% (denoted in the dashed line). This indicates that no cell-killing effect occurs with TiO_2 alone in the dark as well as under visible light irradiation. From these findings the combination of the dye–TiO_2 particles and the polychromatic visible light is effective to kill cancer cells. At 2×10^{-5} mol L^{-1} of Chl-e6, in particular, its anticancer effect and photocytotoxicity is 10 times higher than that of Chl-e6 alone ($p < 0.05$ by Student's *t*-test).

Anticancer effect of the TiO_2 particles is assumed to be caused by the reactive oxygen species generated by the conduction-band electron of TiO_2 produced by dye sensitization. The cell-killing effect and its mechanism is, therefore, similar to those occurring in the previously reported TiO_2-based PDT systems.[1–3] Advantage of our system is high sensitivity of PDT to visible light. Presently, we have not succeeded in identifying the origin for the photocytotoxicity. PDT systems using dye sensitizers involve singlet oxygen as a main reactive species. Previous study with conjugated Chl-e6 sensitizers visualized generation of reactive oxygen species using a fluorescent precursor.[6] We investigated the generation of reactive oxygen species using an ESR spectrometer (JES-AF200, JEOL) with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Reaction of DMPO with reactive oxygen species forms DOPM-OH adduct, which gives the characteristic ESR signal having the four peaks whose intensity ratio is 1:2:2:1.[13] Figures 2A and 2B present the ESR spectra of the dye–TiO_2 particle dispersion including DMPO with and without the irradiation of the polychromatic visible light for an hour, respectively. We observed ESR signals in both systems. The ESR of the nonirradiation control gives a background signal level under the experimental condition. The peak intensity for the light-irradiated sample is amply larger than that of the background as is demonstrated by the differential ESR spectrum in Figure 2C, in which we can exactly identify the ESR signal characteristic to DOPM-OH adduct.

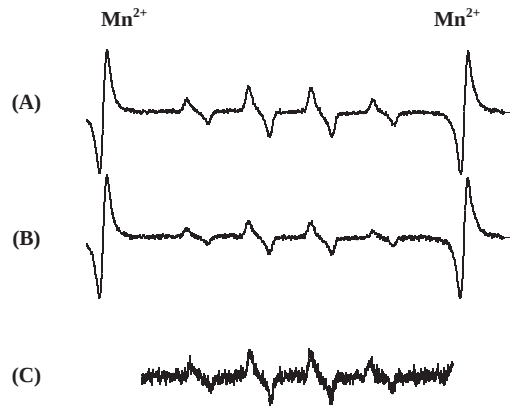

Figure 2. ESR spectra of dye–TiO_2 particle dispersion, (A): with irradiation, (B): without irradiation, and (C): differential ESR spectrum between the spectrum (A) and (B).

Consequently, the anticancer modality consisting of the dye–TiO_2 particles and the polychromatic visible light is significantly effective to damage the EL-4 cells. The observed anticancer effect would be caused by reactive oxygen species, for example, singlet oxygen, superoxide, and hydroxy radical, generated by the photoexcited dye–TiO_2 particles although the kinds of reactive oxygen species involved has not been specified at the present stage. The cell-killing effect by the present method can occur for normal tissues. It is expected, however, that the positively charged TiO_2 nanocrystals[8,9] have higher affinity to tumor cells, which are more negatively charged than normal cells. In vivo experiments in this aspect are now under progress.

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