Effects of TiO\textsubscript{2} Nanoparticles on Cytotoxic Action of Chemotherapeutic Drugs Against a Human Oral Squamous Cell Carcinoma Cell Line

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Abstract. Background. Despite the rapid development of nanotechnology, the biological significance of TiO\textsubscript{2} nanoparticles (NPs), possibly released from dental materials, is not well-understood. We investigated the effect of TiO\textsubscript{2} NPs on the sensitivity of human oral squamous cell carcinoma (OSCC) cell line (HSC-2) to five popular chemotherapeutic agents. Materials and Methods. Viable cell number was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The aggregation and cellular uptake of TiO\textsubscript{2} NPs were assessed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively. Adsorption of TiO\textsubscript{2} NPs to anticancer drugs was assessed by the antitumor activity recovered from the TiO\textsubscript{2} NP-free supernatant. Results: When mixed with culture medium, TiO\textsubscript{2} NPs instantly aggregated, and some particles were incorporated into the cells, exclusively in the vacuoles. TiO\textsubscript{2} NPs showed no cytotoxicity nor hormetic growth stimulation at lower concentrations. Doxorubicin, melphalan, 5-fluorouracil and gefitinib were cytotoxic, whereas docetaxel was cytostatic with or without TiO\textsubscript{2} NPs. TiO\textsubscript{2} NPs, at wide concentration ranges (0.2-3.2 mM), did not significantly affect the adsorption of NPs to any of these anticancer drugs, nor affected their cytotoxic or cytostatic activity. Conclusion: This experimental study demonstrated for the first time that TiO\textsubscript{2} NP do not affect the antitumor potential of chemotherapeutic agents against the HSC-2 OSCC cell line.

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and chondrosarcoma SW1353 cell lines dose-dependently (12) and induced apoptosis of the human lung cancer A549 cell line (13) by its pro-oxidant action. On the other hand, TiO₂ NPs had no apparent cytotoxicity against human kidney glomerular mesangial IP 15 and epithelial proximal HK-2 cell lines, even at high doses (14). Furthermore, how TiO₂ NPs affect the sensitivity of oral cells against dental treatment is not clear.

We investigated whether TiO₂ NPs (i) become aggregated in culture medium; (ii) are incorporated into cells and show cytotoxicity; (iii) adsorb to five popular antitumor agents [doxorubicin (anthracycline antibiotic), melphalan (alkylating agent), 5-fluorouracil (5-FU, pyrimidine analog), docetaxel (taxane), gefitinib (protein tyrosine kinase inhibitor)]; and (iv) modify antitumor action of these agents, in the human oral squamous cell carcinoma (OSCC) cell line HSC-2.

Materials and Methods

**Materials.** The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM), Gibco BRL (Grand Island, NY, USA); fetal bovine serum (FBS), JRH Bioscience (Lenexa, KS, USA); TiO₂ NPs (nanopowder, anatase phase, particle size <25 nm, purity 99.7% trace metal basis, MW 79.87 g/mol) and melphalan Aldrich (St. Louis, MO, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), culture plastic dishes, 6-well and 96-microwell plates, Becton Dickinson (Franklin Lakes, NJ, USA); doxorubicin and dimethyl sulfoxide (DMSO) Wako Pure Chem Ind. (Osaka, Japan); 5-FU, Kyowa (Tokyo, Japan); docetaxel, PKC Pharmaceuticals, Inc. (Woburn, MA, USA); zolium bromide (MTT), culture plastic dishes, 6-well and 96-microwell plates, Becton Dickinson (Franklin Lakes, NJ, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), culture plastic dishes, 6-well and 96-microwell plates, Becton Dickinson (Franklin Lakes, NJ, USA); doxorubicin and dimethyl sulfoxide (DMSO) Wako Pure Chem Ind. (Osaka, Japan); 5-FU, Kyowa (Tokyo, Japan); docetaxel, PKC Pharmaceuticals, Inc. (Woburn, MA, USA).

**Assay for TiO₂ NP topography.** TiO₂ NPs were vortexed and suspended by sonication in distilled water with a bath-type sonicator (Tokyo Cho-onpa Giken Co. Tokyo, Japan) for 1 min at room temperature, since water was found to be the best vehicle to produce a homogeneous suspension of TiO₂ NPs most efficiently, as compared with alcohol, phosphate-buffered saline without calcium and magnesium [PBS(−)] or medium (data not shown). A drop of TiO₂ NPs was set on an aluminum stub, dried for 48 h at room temperature, and covered with 50 nm of gold sputtering. The topographical surface was then observed with scanning electron microscope (SEM) (JSM-6360LV; JEOL, Tachikawa, Japan) with secondary electrons at ×10,000 magnification by 15 kV.

**Cell culture.** HSC-2 cells (Riken Cell Bank, Tsukuba, Japan) were cultured at 37°C in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate under a humidified atmosphere with 5% CO₂. Cells were then harvested by treatment with 0.25% trypsin-0.025% EDTA-2Na in PBS(−) and either subcultured or used for experiments.

**Assay for cytotoxic activity.** Cells (2×10⁴ cells/0.1 ml) were inoculated into each well of 96-microwell plates and incubated for 48 h to achieve the complete cell adherence. The medium was replaced with 0.1 ml of fresh medium containing 0, 0.2, 0.4, 0.8, 1.6 and 3.2 mM of TiO₂ NPs. After 30 min, the cells were treated with different concentrations of doxorubicin, melphalan, 5-FU, docetaxel or gefitinib and incubated further for 48 h. The relative viable cell number was then determined by the MTT method. In brief, the culture medium was replaced with MTT (0.2 mg/ml), and cells were incubated for 4 h at 37°C. After removal of medium, the formazan product was dissolved with DMSO, and the absorbance at 540 nm of the lysate, which reflects the mitochondrial activity, was determined by using a microplate reader (Multiskan; Biochromatic Labsystem, Osaka, Japan) (15). The 50% cytotoxic concentration (CC₅₀) was determined from the dose–response curve and the mean CC₅₀ (±S.D.) value of each anticancer drug was calculated from three independent experiments each of which were performed in triplicate.

**Interaction of TiO₂ NPs with anticancer drugs.** Doxorubicin (final: 1 mM), melphalan (10 mM), 5-FU (10 mM), docetaxel (0.1 mM) or gefitinib (10 mM) was mixed with TiO₂ NPs (final: 8 mM) in a total volume of 40 μl of DMSO, and stood at room temperature for 10 min. The mixture was centrifuged at 21,880 × g for 5 min, and the supernatant, free from TiO₂ NPs, was collected. Near-confluent HSC-2 cells were incubated for 48 h with serially diluted supernatant, and the relative viable cell number was then determined by MTT method as described above and compared with that of treatment without the use of TiO₂ NPs.

**Intracellular uptake of TiO₂ NPs.** Cells were treated with 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mM of TiO₂ NPs for 3 h. The cells were then washed three times with cold PBS(−) and fixed for 1 h with 2% gultaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C, scraped with a rubber policeman, dehydrated and then embedded in Araldite M (Ciba–Geigy Swiss; NISSHIN EN Co., Ltd., Tokyo, Japan). Thin sections were stained with uranyl acetate and lead citrate, and were then observed under a JEM-1210 transmission electron microscope (TEM) (JEOL) (magnification: ×5,000) at an accelerating voltage of 100 kV (16).
Statistical analysis. Data are expressed as the mean±standard deviation (S.D.). Statistical analysis was carried out with Kruskal–Wallis test and multiple comparisons by Mann–Whitney test with SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA). Differences were considered significant at *p*<0.05.

Results

Topography and intracellular uptake of TiO$_2$ NPs. We prepared a homogeneous TiO$_2$ NP suspension by sonication in water. However, TiO$_2$ NPs easily aggregate to make clusters during the preparation for SEM (Figure 1) or when added to culture medium (as demonstrated by TEM) (Figure 2). This may be due to the interactions between the amine and carboxylic groups on the cell membrane (negative charge) and the surface of TiO$_2$ NPs (positive charge) (11). Interaction or attraction between the cells and TiO$_2$ NPs may produce high biocompatibility. Some TiO$_2$ NPs were incorporated into the cells, specifically in the vacuoles, in a dose-dependent fashion (Figure 2).

Possible interaction of TiO$_2$ NPs and anticancer drugs. High affinity of cells for TiO$_2$ NPs prompted us to investigate whether TiO$_2$ NPs show similar affinity toward test compounds. To test this possibility, we first incubated TiO$_2$ NPs with each anticancer drug (doxorubicin, melphalan, 5-FU, docetaxel, or gefitinib) in DMSO, and then collected the supernatant that did not contain TiO$_2$ NPs. If a significant preparation of TiO$_2$ NPs were adsorbed to the anticancer drugs,
Figure 3. Interaction between anticancer drugs and TiO$_2$ nanoparticles (NPs). A: Flow chart of experimental procedure. B: The five anticancer drugs were first mixed with (closed symbols) or without (open symbols) TiO$_2$ NPs (64 mM), and the TiO$_2$-free supernatant was collected after centrifugation at 21,880 ×g for 5 min. Near-confluent HSC-2 cells were then incubated for 48 h with serially-diluted supernatant (0, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2%) to determine the viable cell number by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The highest concentration (2%) of supernatant without pre-incubation with TiO$_2$ NPs contained 10 μM doxorubicin, 100 μM melphalan, 100 μM 5-FU, 1 μM docetaxel and 100 μM gefitinib, respectively. Each value represents the mean±S.D. of triplicate assays.
Figure 4. Effect of TiO$_2$ nanoparticles (NPs) on growth-inhibitory activity of anticancer drugs against HSC-2 cells. HSC-2 cells were first pre-treated for 30 min in the presence of 0, 0.2, 0.4, 0.8, 1.6 or 3.2 mM TiO$_2$ NPs, and then incubated for a further 48 h with doxorubicin (A, F, K), melphalan (B, G, L), 5-fluorouracil (5-FU) (C, H, M), docetaxel (D, I, N) or gefitinib (E, J, O) to determine the viable cell number by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. The data of first (A-E), second (F-J) and third (K-O) experiments are shown. Each value represents the mean±S.D. of triplicate assays. Absorbance value of control cells treated with 0, 0.2, 0.4, 0.8, 1.6 or 3.2 mM TiO$_2$ NPs without anticancer drug was 2.060±0.358 (100%), 1.915±0.332 (93%), 2.039±0.479 (95%), 1.957±0.432 (95%), 2.120±0.399 (103%) and 2.027±0.367 (98%), respectively (n=45, triplicate × three times experiments × five anticancer drugs). There was statistically no difference between 0 and other concentrations of TiO$_2$ NPs (p>0.30). It should be noted that concentration ranges for some compounds were changed in experiments 1 to 3, so as to determine the CC$_{50}$ values.
the concentration of anticancer drugs recovered from the supernatant would be reduced. The results showed this was not
the case, with superimposable dose–response curves, regardless of the presence or absence of TiO2 NPs (Figure 3). These
results show adsorption of TiO2 NPs to anticancer drugs to be low or having little effect on bioavailability of these drugs.

Effect on cytotoxic activity of anticancer drugs. Next, we investigated whether TiO2 NPs modify the cytotoxicity of
anticancer drugs. HSC-2 cells were first pre-incubated for 30 min with TiO2 NPs (0, 0.2, 0.4, 0.8, 1.6 or 3.2 mM), and
then added with increasing concentrations of each anticancer drug and cells incubated for a further 48 h to determine the
viable cell number. We found that TiO2 NPs had no cytotoxicity nor hormetic growth stimulation at lower concentrations (Figure 4). Doxorubicin, melphalan, 5-FU, and gefitinib were cytotoxic, completely killing the cells at higher concentrations, whereas docetaxel was cytostatic, regardless of the presence or absence of TiO2 NPs (Figure 4). The dose-dependent curves of their antitumor activities were superimposable, regardless of the concentration of TiO2 NPs (Figure 4). Repeated experiments confirmed that the CC50 values at any TiO2 NP concentrations were not significantly (p>0.05) different from those of the controls (without TiO2 NPs), maintaining the same order of antitumor potency: docetaxel > doxorubicin > melphanal > gefitinib, 5-FU (Table I). This demonstrates that TiO2 NPs did not potentiate or reduce the antitumor potential of the five anticancer drugs tested.

Discussion

Nanoparticles have a wide applications in the field of medicine and dentistry due to their antibacterial, antifungal and antiviral potential (17-19). However, the study of the antitumor potential of TiO2 NPs has been limited. Recently, it has been reported that TiO2 NPs alone showed little or no toxicity against rat glioma C6, RG2, mouse melanoma B16 and human glioma U373 cell lines, and copper–TiO2 NP complex were significantly less cytotoxic than copper alone, suggesting some protective effect of the complex formation with TiO2 NPs, although its cytotoxicity was slightly higher than that of cisplatin (20). The Copper–TiO2 complex maybe incorporated into mitochondria to reduce ATP synthesis and formation of nitrogenous bases, and reach the cell nucleus to create links with DNA base pairs by interaction or groove binding and finally induce apoptotic cell death (20). It has been also reported that TiO2 NPs doped with Au and Pt effectively killed cells of the human erythroleukemia tumor cell line K562 (21).

The uptake mechanisms for TiO2 NPs are still unclear, however, it has been reported that TiO2 NPs were taken-up and accumulated in the vacuoles, endosomes and lysosomes, or localized in the cytoplasm possibly due to lysosomal membrane rupture (20, 22), confirming the present finding with HSC-2 OSCC cell line that the TiO2 NPs were sequestered only into the vacuoles, where interestingly they became aggregated again (Figure 2). It was recently reported that TiO2 NPs were transported through plasma membrane via transmembrane toll-like receptor (TLR 4) in human hepatocellular carcinoma HepG2 and human chronic myelogenous leukemia K562 cell lines (23). The present study demonstrated that TiO2 NPs, at a wide range of concentrations (up to 3.2 mM), did not adsorb to any of the five anticancer drugs (doxorubicin, melphalan, 5-FU, docetaxel, gefitinib), nor affected their growth–inhibitory activity against the HSC-2 against human OSCC cell line, supporting a previous report with other types of cultured cells (18). The present study demonstrated, to our knowledge, for the first time, that TiO2 NPs themselves exhibit no cytotoxicity nor hormetic growth stimulation at low concentrations, quite differently than many toxic substances, environmental hormones, inorganic compounds, and even irradiation, modulate the growth of cultured cells in a bi-phasic fashion, stimulating or inhibiting the growth

<table>
<thead>
<tr>
<th>TiO2 (mM) concentration</th>
<th>Doxorubicin (μM)</th>
<th>Melphalan (μM)</th>
<th>5-FU (μM)</th>
<th>Docetaxel (μM)</th>
<th>Gefitinib (μM)</th>
</tr>
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<tr>
<td>0</td>
<td>0.23±.04</td>
<td>12.8±5.37</td>
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<td>0.0025±.0014</td>
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<td>0.2</td>
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<td>17.8±9.50</td>
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<td>0.0033±.0023</td>
<td>31.84±16.95</td>
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<tr>
<td>0.4</td>
<td>0.19±.003</td>
<td>12.8±4.22</td>
<td>42.0±11.83</td>
<td>0.0027±.0024</td>
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<tr>
<td>0.8</td>
<td>0.21±.05</td>
<td>14.7±6.50</td>
<td>25.5±1.16</td>
<td>0.0035±.0022</td>
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<tr>
<td>1.6</td>
<td>0.22±.04</td>
<td>11.6±4.47</td>
<td>17.9±7.70</td>
<td>0.0040±.0009</td>
<td>31.27±3.98</td>
</tr>
<tr>
<td>3.2</td>
<td>0.19±.08</td>
<td>13.7±4.92</td>
<td>38.6±16.06</td>
<td>0.0061±.0036</td>
<td>30.93±5.94</td>
</tr>
</tbody>
</table>

CC50: Cytotoxic concentration; S.D.: standard deviation; Kruskal-Wallis and multiple comparisons by Mann-Whitney did not show any significant differences (p>0.05) by TiO2 concentrations.
of cultured cells at lower and higher concentrations, respectively, as seen in previous studies (24).

TiO₂ NPs slightly reduced the cytostatic activity of docetaxel (Table I), however, the CC₅₀ values varied considerably due to the cytostatic (not cytotoxic) property of this compound. Taken together with the previous reports, we conclude that TiO₂ NPs showed no cytotoxicity nor hormetic growth stimulation at lower concentrations, nor affected the antitumor potential of chemotherapeutic agents when used against of OSCC cell line HSC-2. However, this would not mean that TiO₂ NPs are absolutely safe substances in the oral environment, since TiO₂ NPs aggravated inflammation induced by interleukin-1β in human gingival fibroblast (unpublished data). The interaction between OSCC and inflammatory human gingival fibroblast cells in the oral cavity remains to be investigated.

References