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Acute effects of sono-activated photocatalytic titanium dioxide nanoparticles on oral squamous cell carcinoma

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ABSTRACT

Sonodynamic therapy (SDT) is a new treatment modality using ultrasound to activate certain chemical sensitizers for cancer therapy. In this study, effects of high intensity focused ultrasound (HIFU) combined with photocatalytic titanium dioxide (TiO_2) nanoparticles on human oral squamous cell line HSC-2 were investigated. Viability of HSC-2 cells after 0, 0.1, 1, or 3 s of HIFU irradiation with 20, 32, 55 and 73 W cm^{-2} intensities in the presence or absence of TiO_2 was measured immediately after the exposures *in vitro*. Immediate effects of HIFU (3 s, 73 W cm^{-2}) combined with TiO_2 on solid tumors were also examined by histological study. Cytotoxic effect of HIFU + TiO_2 *in vitro* was significantly higher than that of TiO_2 or HIFU alone with the tendency to increase for higher HIFU intensity, duration, and TiO_2 concentration in the suspension. *In vivo* results showed significant necrosis and tissue damage in HIFU and HIFU + TiO_2 treated samples. However, penetration of TiO_2 nanoparticles into the cell cytoplasm was only observed in HIFU + TiO_2 treated tissues. In this study, our findings provide a rational basis for the development of an effective HIFU based sonodynamic activation method. This approach offers an attractive non-invasive therapy technique for oral cancer in future.

1. Introduction

Oral squamous cell carcinoma (OSCC), originating in squamous cells that line the mouth and lips, makes around 90% of all oropharyngeal malignancies. Oral cancer is ranked 11th among the top most common cancers worldwide with higher prevalence in developing countries. Although smoking and excessive alcohol consumption are prominent oral cancer risk factors, human papillomavirus (HPV) infection and population aging have greatly contributed to the increased cancer incidents in recent years. Despite advances in cancer diagnosis and treatment, OSCC still is among the tumor types with a poor prognosis. The survival rate of the patients at 5 years from diagnosis stands at about 50%, which is associated with the cancer being discovered late in its development [1]. Depending on the tumor size, lymph node involvement and spread of the cancer, surgery in combination with chemotherapy and/or radiotherapy is the primary treatment for OSCC [2]. Surgery usually involves radical removal of the malignant tissue along with a large portion of the adjacent structures containing ancillary lymph nodes that drain the tumor and may cause metastasis. Consequently, many of these patients should undergo reconstructive surgeries to restore their facial appearance or to help them regain the ability to talk and eat. Side effects associated with conventional oral cancer treatment, especially in advanced cases, can significantly impair quality of life (QOL) of the patients and compromise the continuity of cancer treatment. Although traditional treatment remains the treatment of choice for many cases of OSCC at early stages, concerns over unwanted side effects following damages to the normal tissue have now become an important issue in the assessment of treatment strategies for advanced oral cancer [3]. As a result, there has been a considerable shift in the cancer therapies toward minimally invasive techniques for precisely targeting OSCC cells [4,5].

In recent years, photochemical reactions for treating tumors have drawn much attention. Photodynamic therapy (PDT) has been used clinically to treat a wide range of medical conditions, including malignant tumors and abnormal vasculature which are accessible to an activating light [6,7]. PDT uses light absorbing photosensitizer to generate highly reactive oxygen species which can cause localized cytotoxicity. Despite great advantages of PDT

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for cancer treatment, its implications in the clinical situations are limited only to superficial tumors, owing to the short penetration distance of the light. Moreover, the patients are photosensitive for several weeks following systemic administration of the photosensitizer. Replacing the source of light in PDT with ultrasound (US) has emerged as a promising method for treatment of tumors deep seated in the body. Sonodynamic therapy (SDT) consists of uptake and/or retention of a sonosensitizing drug (sonosensitizers) in tissues and subsequent activation of the drug upon interaction with US [8,9].

Among various photo/sonocatalyst materials, TiO_2 is the most used semiconductor with high catalytic activity, non-toxicity, low cost and photo/sonochemical stability in the reaction condition [10–18]. While widely applied for industrial wastewater purification to degrade harmful chemicals and inactivate microorganisms [19–23], TiO_2 has been an attractive catalyzer for treating a variety of malignant tumors, including oral cancers, owing to its strong oxidizing activity, chemically unreactive in biological systems and easy to prepare in stable suspensions in nano-particulate form [24–28]. Extensive studies of TiO_2 nanoparticles have shown generation of hydroxyl radicals under ultrasound radiation subsequently causing cell death [29–31].

With regard to its potential, SDT can play an increasingly important role as a targeting technique in cancer treatment. Nevertheless, there are challenges to overcome before its clinical application becomes a reality. One of the critical areas for clinically applying SDT is maximizing TiO_2 delivery to the cells *in vivo*. Despite encouraging *in vitro* reports, translating the effects of SDT *in vivo* as a therapeutic modality for an accurate and safe OSCC treatment remains a challenge, which is the subject of the present study. Here, we investigated *in vitro* and *in vivo* effects of HIFU combined with TiO_2 nanoparticles on oral cancer cells to determine whether HIFU can be used as a physical tool to transfer the nanoparticles in the tumor cells *in vivo*. The results pave the way for potential application of HIFU based sonodynamic activation method.

2. Material and methods

2.1. Cell cultures

Oral squamous carcinoma cell line HSC-2 purchased from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank) cultured in MEM medium (Wako, Osaka, Japan) with 10% Fetal Bovine Serum (Invitrogen Co., Tokyo, Japan) was maintained at 37.0 °C in humidified air with 5% CO_2. HSC-2 cells collected by trypsin–EDTA (Gibco, NY, USA) were washed and maintained in fresh medium immediately before each experiment. Cells with initial viability of more than 99% were used for the experiments.

2.2. Animals

BALB/c athymic nude mice obtained from Central Institute for Experimental Animals (CIEA, Tokyo, Japan) were housed in a controlled environment at 22 °C on a 12 h dark cycle with free access to food and water. They were 7–8 weeks old at the beginning of the experiments, weighing 20–25 g. All animal studies were carried out with the approval of Fukuoka University Experimental Animal Care and Use Committee. HSC-2 cell suspension of 2×10^6 in 0.1 ml PBS were subcutaneously injected into the right flanks of nude mice after the mice were anesthetized with diethyl ether (Sigma–Aldrich, Tokyo, Japan).

2.3. Titanium oxide (TiO_2)-silica aqueous

Titania-silica aqueous solution (TiO_2) used as sonocatalyst in this experiment is prepared by coupling titanium oxide and silicon oxide with hydrogen peroxide (Asaka Riken Co., Ltd., Fukushima, Japan). It has a peroxo-titania-silica type (R-P-TS) and an anatase-titania-silica type (R-A-TS). Peroxo-titania-silica (R-P-TS) solution contains 0.028–0.030% peroxide on a hydrogen peroxide conversion basis, 2.8–3.9% peroxide per solid weight. Anatase-titania-silica (R-A-TS) solution, on the other hand, contains 0.0008–0.0010% peroxide on a hydrogen peroxide conversion basis, maximum of 0.1% peroxide per solid weight. Both solutions contain TiO_2 nanoparticles with an average size of about 10–30 nm in diameter, stable at room temperature with no tendency of aggregation within biological fluids used in the experiment.

2.4. HIFU sonication protocol

A single-element 3.5 MHz HIFU transducer (Nepagene, Chiba, Japan) connected to an ultrasound generator (SonoPore KTAC-4000, 0–60 V setting, Nepagene, Chiba, Japan) was used to generate the high-intensity focused ultrasound. The transducer had 30 mm aperture diameter and 50 mm radius of curvature (focal length), shown in Fig. 1. The ultrasound pressure field was measured by a PVDF needle hydrophone (0.5 mm diameter pressure sensor, 40 ns rise time, 1.29 mV/bar sensitivity, Müller Ingenieurtechnik, Germany). The hydrophone was tested and calibrated with a fiber optic probe hydrophone (100 μm diameter and 3 ns rise time) (FOPH 2000, RP acoustics, Germany). The pressure measurements were performed in a large test tank with a rubber absorber panel facing the transducer to eliminate any wave reflection. The HIFU transducer's peak focal pressures were 0.77, 1.0, 1.35, 1.56 MPa positive (P^+) and −0.77, −0.94, −1.2, −1.38 MPa negative (P^-) (Fig. 2); corresponding to spatial-peak pulse-average intensities 20, 32, 55, and 73 W cm^{-2}. The transducer had 0.8 mm lateral diameter and 11 mm axial length of focal extension at −6 dB (full-width at half-maximum FWHM) pressure. Therefore, the transducer's output acoustic powers were 50, 80, 140 and 200 mW at 30, 40, 50, and 60 V driving voltages, respectively. The same HIFU transducer was used for both *in vitro* and *in vivo* experiments. Temperature changes immediately after HIFU exposures were less than 1.0 °C, which agrees with estimated 0.27 °C temperature rise of the 500 μl cell suspension (specific heat of 4200 J kg^{-1} $°C^{-1}$) after 3 s of 200 mW (maximum) acoustic power exposure.

2.5. In vitro treatment protocol

Suspend HSC-2 cells in fresh medium (9×10^4 cells/ml) were divided into four groups; control, HIFU treated, TiO_2 treated and combination of HIFU + TiO_2 treated groups. Cell suspension of 500 μl/well volume was placed in 24-well Lumox® plate (SARSTEDT AG & Co., Germany). Each well has a 50 μm thick film bottom which is gas permeable and acoustically transparent. Titania-Silica (R-A-TS) was added right before the experiments at the final concentration of 0.01%, 0.03% and 0.06% (v/v) to cell suspension of TiO_2 treated and combination of HIFU + TiO_2 group. For HIFU treatment, bottom of the plate was placed on top of the HIFU transducer with degassed water filling the space between the transducer surface and the plate, shown in Fig. 1. Custom-made cylindrical acoustic absorbers (EUA101A, Eastek Corp Japan, >40 dB at 1 MHz) were placed in the wells to reduce ultrasound reflection, nesting directly on the surface of the cell suspension with no air pockets remaining between them. Cell suspension was exposed to variety of HIFU intensities (20, 32, 55, and 73 W cm^{-2}) for 0.1, 1, and 3 s durations. Cell viability was mea-