Photodynamic Effects of Radachlorin® on Cervical Cancer Cells

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Purpose: Photodynamic therapy (PDT) is a novel treatment modality, which produces local tissue necrosis with laser light following the prior administration of a photosensitizing agent. Radachlorin® has recently been shown to be a promising PDT sensitizer. In order to elucidate the antitumor effects of PDT using Radachlorin® on cervical cancer, growth inhibition studies on a HPV-associated tumor cell line, TC-1 cells in vitro and animals with an established TC-1 tumor in vivo were determined.

Materials and methods: TC-1 tumor cells were exposed to various concentrations of Radachlorin® and PDT, with irradiation of 12.5 or 25 J/cm² at an irradiance of 20 mW/cm² using a Won-PDT D662 laser at 662 nm in vitro. C57BL/6 mice with TC-1 tumor were injected with Radachlorin® via different routes and treated with PDT in vivo. A growth suppression study was then used to evaluate the effects at various time points after PDT.

Results: The results showed that irradiation of TC-1 tumor cells in the presence of Radachlorin® induced significant cell growth inhibition. Animals with established TC-1 tumors exhibited significantly smaller tumor sizes over time when treated with Radachlorin® and irradiation.

Conclusion: PDT after the application of Radachlorin® appears to be effective against TC-1 tumors both in vitro and in vivo. (Cancer Research and Treatment 2004;36: 389-394)

Key Words: Radachlorin®, Photodynamic therapy (PDT), Cervical cancer, TC-1 cell
have only been a few studies on the PDT effects of Radachlorin® in cervical cancer, although there have been several studies on Chlorin e₆, which is a major component of Radachlorin®. Therefore, in this study, the PDT induced antitumor effects of Radachlorin® were evaluated in cervical cancer cells and an animal model.

MATERIALS AND METHODS

1) Photosensitizer

The Radachlorin® was purchased from the RADA-PHARMA group (RADA-PHARMA Co, Ltd., Moscow, Russia), which was stable in solutions at 0±8°C in the dark.

2) Cell culture conditions

A mouse lung cancer cell line of TC-1 cells, which was derived from primary epithelial cells of C57BL/6 mice co-transformed with HPV-16 E6 and E7, as well as c-Ha-ras oncogenes (from Cancer Research Center, Seoul National University, Korea), were cultured on RPMI 1640 media (Gibco BRL, Rocksville, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), Streptomycin/penicillin (Gibco BRL), L-glutamine (Gibco BRL), 2.2 mg/ml sodium bicarbonate (Sigma, St. Louis, MO) 0.4 mg/ml G418 disulfate (Duchefa, Netherlands) were added to the culture medium and the cells maintained at 37°C in a 5% CO₂ humid environment.

3) Immunization of mice

The female C57BL/6 mice (6-8 weeks old) were purchased from DaeHan Biolink (Daejon, Korea), and maintained under pathogen-free conditions. A TC-1 tumor animal model was established as previously reported (22). Briefly, 0.1 ml PBS suspension (3×10⁴ cells/ml) of TC-1 cells was injected subcutaneously into the belly of the mice using a syringe. After the cancer cells had made a tumor size of 9 mm, the TC-1 cells were washed twice with PBS and fixed with 1% paraformaldehyde. The cells were then washed again with distilled water, the cover glasses removed from the 6 well plates and mounted on slide glass. Confocal microscope (MRC 1024, Bio-RAD, Hercules, CA) measurements were performed at emission and excitation wavelengths of 545 and 600 nm.

6) MTT assay

TC-1 cell lines were inoculated into a 96-well, flat-bottomed microplate at a volume of 100 μl (2×10⁴ cells/well) for a stationary culture. Twenty-four hours later, the medium was removed, and the cultures washed three times in PBS. Various concentrations (0, 2.5, 5, 10 and 20 μg/ml) of Radachlorin® were then added in a volume of 100 μl/well. The cultures were then subjected to laser irradiation (12.5 or 25 J/cm²), followed by the MTT assay to evaluate their sensitivity to PDT (Radachlorin®). For the MTT assay, 20 μl of MTT reagent (5 mg/ml) was added to each cell culture well and cultured for 4 h. 200 μl of DMSO was added to the culture, shaken for 10 min and the absorbance measured with an ELISA-reader at 570 nm. Measurements were performed for 6 days after the laser irradiation. Samples were assayed in triplicate, and the mean used as the measured value. The amount of Radachlorin® was also compared with the cancer cell lines.

7) Inhibition of TC-1 tumor growth in vivo

Animals were randomized into four groups (ten animals in each group): (●) control (untreated); (△) Radachlorin® only; (▲) irradiation only; (○) Radachlorin® 40 mg/kg b.w. intravenous (i.v.) injection and irradiation; (●) Radachlorin® 40 mg/kg b.w. intraperitoneal (i.p.) injection and irradiation. The TC-1 cell implanted mice were either i.v. or i.p. injected with 40 mg of Radachlorin®/kg of b.w., respectively, and PDT performed.

4) PDT

The PDT was carried out using a laser apparatus generated by a diode (Won-PDT D662, Won Technology, Daejeon, Korea) equipped with high power laser diode module, with a built in temperature control system, optical fiber bundle and fiber test module. The wavelength was set at 662±3 nm. The duration of the light irradiation, under PDT treatment, was calculated taking into account the empirically found effective dose of light energy in J/W.

5) Radachlorin® uptake by TC-1 cells in vitro

TC-1 cells were inoculated into 6 well plates, with cover glasses, in a volume of 2 ml (5×10⁴ cells/well) for a stationary culture. Twenty-four hours later, Radachlorin® (2.5, 5, 10, 20 and 50 μg/ml) was added in a volume of 2 ml. After a predetermined time, the Radachlorin® solution was discarded; the TC-1 cells were washed twice with PBS and fixed with 1% paraformaldehyde. The cells were then washed again with distilled water, the cover glasses removed from the 6 well plates and mounted on slide glass. Confocal microscope (MRC 6 )  MTT assay

TC-1 cell lines were inoculated into a 96-well, flat-bottomed microplate at a volume of 100 μl (2×10⁴ cells/well) for a stationary culture. Twenty-four hours later, the medium was removed, and the cultures washed three times in PBS. Various concentrations (0, 2.5, 5, 10 and 20 μg/ml) of Radachlorin® were then added in a volume of 100 μl/well. The cultures were then subjected to laser irradiation (12.5 or 25 J/cm²), followed by the MTT assay to evaluate their sensitivity to PDT (Radachlorin®). For the MTT assay, 20 μl of MTT reagent (5 mg/ml) was added to each cell culture well and cultured for 4 h. 200 μl of DMSO was added to the culture, shaken for 10 min and the absorbance measured with an ELISA-reader at 570 nm. Measurements were performed for 6 days after the laser irradiation. Samples were assayed in triplicate, and the mean used as the measured value. The amount of Radachlorin® was also compared with the cancer cell lines.

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RESULTS

1) Observation of Radachlorin® uptake by TC-1 cells

Fig. 2 shows the confocal microscopy of TC-1 cells after 24 h exposure to various concentrations of Radachlorin®. TC-1 cells were seen to contain Radachlorin®, which was excited to emit red to a confocal microscope. The luminescence of each cell was higher, in a Radachlorin® dose dependent manner (A-F). The Radachlorin® in the TC-1 cells showed no cytotoxicity, even with a higher concentration of 50 μg/ml (data not shown).

2) Intracellular localization of Radachlorin®

It is important to determine the biological mechanism of action of a drug; therefore, the intracellular distribution of Radachlorin® was determined in TC-1 cells. The intracellular localization of TC-1 cells after 12 h incubation with 5 μg/ml of Radachlorin® was measured by confocal microscopy (Fig.
3). The fluorescence was emitted from well-defined spots in the cytoplasm, and diffused fluorescence seen in the entire cytoplasm. The fluorescence micrographs suggested association of with the plasma membrane.

3) Antitumor effect of PDT using Radachlorin® in vitro

The efficacy of cell damage after PDT with Radachlorin® was further quantified by the MTT assay. The results of the experiment with TC-1 cells are shown in Fig. 4 (A-D). TC-1 cells incubated with various concentration of Radachlorin® and irradiated with laser showed significantly reduced cell viability with increasing light dose (B). However, when TC-1 cells were incubated with 2.5 μg/ml of Radachlorin® for 3 h, and then irradiated with 25 J/cm², the cell viability increased compared to the other Radachlorin® dosed cells (A). At the lowest light dose, 12.5 J/cm², this experiment induced an increased cell viability when Radachlorin® was dosed at 2.5 μg/ml and incubated for 3 and 12 h (C & D). Even though with a lower light dose exposure (12.5 J/cm²), the cell viability was significantly lower with an exposure time of Radachlorin® of 24 h than with 3 or 12 h (data not shown). Therefore, the optimal experimental drug dose of Radachlorin® seems to be 2.5 μg/ml for 3 h or 12 h, with irradiation of 12.5 or 50 J/cm².
Fig. 4. Cell growth-inhibitory effects of PDT on TC-1 cells \textit{in vitro}. Cells (2×10^3 cells/well) were cultured overnight in 96-well plates, in triplicate, and incubated with Radachlorin® for 3 or 12 h, with irradiation of 12.5 or 25 J/cm² at an irradiance of 20 mW/cm² using a Won-PDT D662 laser at 662 nm. After PDT, the cells were cultured for a predetermined time, and the MTT assay performed. The conditions for the TC-1 cells were (A) Radachlorin® 3h incubation, and irradiation 25 J, 20 mW (B) 12 h, and 25 J, 20 mW (C) 3 h, and 12.5 J, 20 mW (D) 12 h, and 12.5 J, 20 mW; ●, Control; ○, 2.5 μg/ml of Radachlorin®; ■, 5 μg/ml of Radachlorin®; □, 10 μg/ml of Radachlorin®; ▲, 20 μg/ml of Radachlorin®.

4) Measurement of Photodynamic effects \textit{in vivo}

The antitumor activity of PDT using Radachlorin® in C57BL/6 mice with TC-1 tumors was determined, as shown in Fig. 5. In the Irradiation and Radachlorin® only group, the tumor sizes increased over the time period. It was observed that the control group, which showed a linear increase in tumor size over the time, was similar. The PDT only group showed no cytotoxicity in the TC-1 tumor lesions. Radachlorin® itself also had no toxicity on mice (data not shown). In the PDT using Radachlorin® treatment group, when the C57BL/6 mice with TC-1 tumors were PDT irradiated using 40 mg of Radachlorin®/kg b.w. (i.p.), the tumor size was significantly reduced compared to the other experimental and PDT using 40 mg of Radachlorin®/kg b.w. (i.v.) groups. PDT with an i.p. injection of Radachlorin® group showed improved antitumor effects over those with an i.v. injection.

\textbf{DISCUSSION}

Radachlorin® has recently been shown to be a promising PDT sensitizer (23), with a report showing the photodynamic effect on novel chlorin e₆ derivatives, including Radachlorin®, on a single nerve cell (24). The study demonstrated that Radachlorin® was a most potent photosensitizer, comparable with \textit{Meso}-[tetrakis(m-hydroxyphenyl)]chlorin (mTHPC), a well-known photosensitizer (24). In this study, TC-1 cells were shown to contain Radachlorin® in a dose dependent manner, which did not affect the viability of cells compared with the values of non-Radachlorin incubated cells (data not shown). Diffused fluorescence was found in the entire cytoplasm. Localization of Radachlorin® uptake by TC-1 cells was not studied in detail in the present study. A previous report has shown that localization of intracellular photosensitizer depends on the lipophilicity and amphiphilicity of the photosensitizer (25). When incubated with cells, molecules of the photosen-
Demonstrated that Radachlorin® photosensitizers available (www.radapharma.ru). Our data also indicate that Radachlorin® is more efficient photosensitizer upon irradiation than the other photosensitizers. Thus, the accumulation of Radachlorin® in PC12 cell lines without irradiation, except with very high concentration, was seen to be adsorbed into the entire cytosol.

In a previous study, a PC12 (pheochromocytoma) cell line and the MTT test were used for in vitro assays, and laser light of 662 nm for Radachlorin® at the doses of 50 J/cm² was irradiated (21). Radachlorin® did not show toxicity on the PC12 cell lines without irradiation, except with very high concentrations. Thus, in vitro Radachlorin® is a less toxic and more efficient photosensitizer upon irradiation than the other photosensitizers available (www.radapharma.ru). Our data also demonstrated that Radachlorin® treatment showed no cytotoxicity on TC-1 cells (data not shown), which supports the findings from previous experiments (21). In the irradiation treated group without Radachlorin®, the accumulation also had no antitumor effect on mice with TC-1 tumors, as shown in Fig. 5. Therefore, these data suggested that Radachlorin® was seen to be adsorbed into the entire cytosol.

In vivo studies (21) demonstrated that Radachlorin® might have significant advantages in the selectively killing of tumor lesions in TC-1 tumors, both in vitro and in vivo.

REFERENCES


