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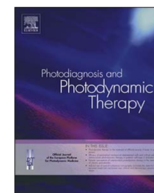

Novel potential photodynamic therapy strategy using 5-Aminolevulinic acid for ovarian clear-cell carcinoma

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ABSTRACT

Background: Photodynamic therapy (PDT) is known as a minimally invasive treatment for cancer. 5-Aminolevulinic acid (ALA) is a precursor of the photosensitizing agent protoporphyrin IX (PpIX). Patients with ovarian clear-cell carcinoma (CCC) have poorer prognoses than those of patients with other histological CCC types. We evaluated the efficacy of ALA-PDT on CCC cells in vitro.
Methods: We used seven human CCC cell lines to measure the cytotoxicity of ALA-PDT. PpIX production in cancer cells was measured using a micro-plate reader. Quantitative real-time PCR was performed to assess the mRNA levels of genes involved in the accumulation of PpIX in cancer cells. Additionally, we measured the enhancement in cytotoxicity with the use of an ABCG2 inhibitor.
Results: We found that three cell lines were highly sensitive to ALA-PDT. In contrast, one cell line was resistant to ALA-PDT. The cytotoxicity of ALA-PDT varied among CCC cell lines. The IC50 values of ALA-PDT for the CCC cell lines had a wide range (30–882 μM). The cytotoxicity of ALA-PDT was correlated with the intracellular PpIX accumulation. The cell lines sensitive to ALA-PDT expressed PEPT1 (an ALA uptake transporter). The cell line resistant to ALA-PDT expressed ABCG2 (a PpIX export transporter). In the resistant cell line, a combination treatment with both ALA and an ABCG2 inhibitor resulted in the promotion of cytotoxic sensitivity.
Conclusion: The present study revealed the efficacy of ALA-PDT against CCC with chemoresistance in vitro.

1. Introduction

It is well known that ovarian cancer is the leading cause of tumor-related death in gynecological cancer [1]. Early-stage ovarian cancer has few subjective symptoms because the ovaries are in the pelvis. Thus, approximately 50% of women with the disease are in an advanced stage when diagnosed. Nearly all patients with advanced disease undergo cytoreductive surgical resection followed by chemotherapy [2]. Clear-cell carcinoma (CCC) is a histological subtype that accounts for 23.4% of all ovarian cancer in Japan [3]. Ovarian CCC has a low response rate to platinum-based chemotherapy; thus, it is associated with a poorer prognosis than that of serous [4,5] and endometrioid adenocarcinoma [6].

Photodynamic therapy (PDT) is an effective and minimally invasive treatment for cancer. It has been reported that some factors are involved in the efficacy of PDT. PDT is generally based on the photochemical interaction of a photosensitizer that can accumulate specifically in cancer tissue with light and oxygen. The type of photosensitizer, light dose, and photosensitizer concentration are also factors related to the effect. Several authors have described that PDT has two types of reactions. The type I reaction results in hydrogen or electron transfer between the photosensitizer and substrate to produce free radicals. These radicals react with oxygen to yield superoxide radical anions. In the type II reaction, singlet oxygen is produced through an energy transfer process with ground state oxygen and the return of the photosensitizer to the ground state [7]. As a representative mechanism, PDT results in the generation of reactive oxygen species (ROS) containing singlet oxygen, which induces apoptosis [8]. There are also reports indicating that an immune response is involved in the effect of PDT. PDT can induce tumor death and microangiopathy, which lead to the increased expression of pro-inflammatory cytokines and the activation of immune cells [9].

In Japan, Photofrin® and Laserphyrin® have been used as photosensitizers in PDT covered by insurance for lung and esophageal cancer.

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The clinical application of PDT is expected to expand to include other cancer types. In terms of PDT, only Photofrin® with an excimer-dye laser was approved for use in treating cervical intraepithelial neoplasia (carcinoma in situ); however, this type of laser is no longer produced, and the maintenance support period ended in March 2017. These photosensitizers have a side effect, i.e., phototoxic skin reactions [10].

In this study, we used 5-aminolevulic acid (ALA), which is water soluble and administered orally [11]. The substance is an endogenous amino acid, and it is usually synthesized from glycine and succinyl CoA in mitochondria. In addition, ALA is not a photosensitizer, it is the precursor of protoporphyrin IX (PpIX) in the heme metabolic pathway. ALA is converted to the active photosensitizer PpIX within cells [12]. PpIX is excreted from the body after 24–48 h via the heme metabolic pathway; therefore, patients do not need to avoid light for a long time [13]. In cancer cells, the irradiation of PpIX with red light at a wavelength of 635 nm induces the generation of ROS, such as singlet oxygen and free radicals, which subsequently cause apoptosis and necrosis [14,15]. Although ingested exogeneous ALA is converted to heme in normal cells, cancer cells specifically accumulate PpIX without metabolizing it to produce heme. In addition, the intracellular accumulation of PpIX was found to involve the solute carrier (SLC) transporter and ATP-binding cassette (ABC) transporter [16–18].

In the present study, we examined the cytotoxicity of ALA-PDT in CCC cells. Furthermore, we explored the expression levels of transporters involved with the cytotoxicity of ALA-PDT and the accumulation of PpIX. The possible application of this modality as a therapy for CCC is proposed.

2. Materials and methods

2.1. Biochemicals and cell culture

ALA hydrochloride was obtained from SBI Pharmaceuticals Co., Ltd. (Tokyo, Japan). We used seven human ovarian CCC cell lines in this study: ES2, TOV21G, KOC7C, OVTOKO, RMG1, RMG2 and OVMANA. ES2 and TOV21G cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). KOC7C cells were a generous gift from Dr. Junzo Kigawa (Tottori University, Tottori, Japan). OVTOKO, RMG1, RMG2, and OVMANA cells were a generous gift from Dr. Kiyoshi Hasegawa (Fujita Health University, Aichi, Japan). The cell lines were maintained in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Light sources for PDT

A light-emitting diode (LED) was used as a source of light at a wavelength of 631 nm. A power density of 17.4 mW/cm^2 was used in this study.

2.3. Cytotoxicity of ALA-PDT

We evaluated the cytotoxicity of ALA-PDT on CCC cells. Cell viability was measured by Cell Counting Kit 8 assay (CCK-8; Dojindo Laboratories, Kumamoto, Japan). ES2, TOV21G, KOC7C and OVTOKO cells were seeded at a density of 1×10^4 cells in 100 μl of media in 96-well plates, and RMG1, RMG2 and OVMANA cells were seeded at a density of 2×10^4 cells in 100 μl of media in 96-well plates. The cells were cultured in an atmosphere with 5% CO_2 at 37 °C for 24 h. The culture media was replaced with fresh media containing ALA at 0–1000 μM. Cells were incubated with ALA in the dark for 4 h. Then, the cells were washed in phosphate-buffered saline (PBS), and the PBS was replaced with fresh media prior to light irradiation. Irradiation with 631-nm light was performed for 600 s. After irradiation, the cells were incubated at 37 °C with 5% CO_2 for 24 h. The cells were washed in PBS before the culture media was replaced with 100 μl of 10% CCK-8 reagent. After the cells were incubated at 37 °C with 5% CO_2 for 30 min, the absorbance at 450 nm was measured using a micro-plate reader (Tecan infinite M200PRO, Zurich, Switzerland). We also prepared a control group without irradiation to evaluate the cytotoxicity of ALA.

2.4. PpIX accumulation in cancer cells incubated with ALA

We evaluated the intracellular and extracellular accumulation of PpIX in CCC cells incubated with ALA. ES2, TOV21G, KOC7C and OVTOKO cells were seeded at a density of 1×10^4 cells in a 100-μl volume in 96-well black wall plates, and RMG1, RMG2 and OVMANA cells were seeded at a density of 2×10^4 cells in a 100-μl volume in 96-well black wall plates. These cells were cultured at 37 °C with 5% CO_2 for 24 h. The culture media was replaced with fresh media containing ALA at various concentrations. The final concentration of ALA ranged from 0 to 1000 μM. The cells were incubated with ALA in the dark for 4 h. Extracellular PpIX was measured in the supernatant. Intracellular PpIX was measured in cells processed with 100 μl of 1% sodium dodecyl sulfate after being washed twice with PBS. The resulting fluorescence was measured using a micro-plate reader (Tecan Infinite M200 PRO). We used an excitation wavelength of 405 nm and emission wavelengths of 632 nm and 635 nm for measuring intracellular and extracellular PpIX accumulation, respectively. The measured values of PpIX accumulation were corrected using protein measurements. The protein concentration was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

2.5. Detection of mRNA levels by quantitative real-time PCR (qRT-PCR)

Total RNA extraction was performed with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracted RNA was immediately stored at −80 °C. The RNA concentration was determined using a spectrophotometer (Beckman Coulter, Pasadena, CA, USA). Transcription was performed using a High-capacity RNA-to-cDNA kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. To obtain complementary DNA (cDNA), 2 μg of RNA was used, and the resulting cDNA was stored at −20 °C. The RNA levels of PEPT1 (SLC15A1), PEPT2 (SLC15A2), TAUT (SLC6A6), GAT2 (SLC6A13), PAT1 (SLC36A1), ABCG2, ferrochelatase (FECH) and β-actin were determined by qRT-PCR (Applied Biosystems® Step One Plus™) with a SYBR Select Master Mix (Applied Biosystems®). β-Actin was used as the internal control, and 20 ng of cDNA was used for qRT-PCR. The cycling conditions consisted of a 2-min hot start at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s, extension at 95 °C for 15 s, and then a final inactivation at 95 °C for 15 s. Relative quantification was performed using the ΔΔCT method. The specific primers for each gene are shown in Table 1. All experiments were performed in triplicate.

2.6. The enhancing effect of ALA-PDT and the accumulation of intracellular PpIX via an ABCG2 inhibitor

We examined the role of ABCG2 in the cytotoxicity induced by ALA-PDT and the accumulation of PpIX. We investigated the inhibitory effect of ABCG2 with an ABCG2-specific inhibitor, fumitremorgin C (FTC). ES2 cells were incubated with ALA (0–1000 μM) for 4 h in the absence or presence of 10 μM FTC according to previously reported methods with some modifications [19,20]. The PDT treatment was followed by cell viability measurements. For the PpIX measurements, ES2 cells were incubated with ALA (0–1000 μM) and FTC (10 μM) in the dark for 4 h. We then measured the intracellular and extracellular PpIX levels according to the abovementioned procedure.

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