Abstract. 5-Aminolevulinic acid-mediated photodynamic therapy (ALA-PDT) is a new ablation treatment for tumors, while its function mechanism in cervical cancer has not been elucidated. In this study, we investigated the effects of ALA-PDT on cervical cancer cell line Me180, to search for optimal parameters of PDT. ALA-PDT on proliferation of Me180 was examined by MTT assay to find the optimal function parameters of ALA-PDT. Apoptosis was observed by using AnnexinV-FITC/PI double staining, Hoechst 33342 staining and May-Grünwald-Farbstoff Giemsa staining. Furthermore, we established a tumor model and 6 mice of each group underwent measurement of the tumor size on days 3, 7, 14, and 21 after treatment. The mRNA expression of survivin, bcl-2, p53, bax and bad in Me180 cells were detected by real-time fluorescence reverse transcription-polymerase chain reaction (RT-PCR) in vitro and in vivo. Finally, we compared the effects between topical and intravenous administration. Based on the above studies, we found ALA-PDT induced apoptosis and G0-G1 phase arrest of Me180 cells. The tumor volume of the topical administration and PDT group was the smallest at 7-14 days post-PDT. H&E staining showed remarkable subcutaneous necrosis in the PDT groups. The mRNA expression of survivin and bcl-2 in Me180 cells were suppressed post-PDT. Topical administration of PDT is recommended in treating cervical cancer so as to minimize the side-effects and inconvenience of phototoxic reaction brought by PDT. Our data may contribute to making the mechanism of PDT on cervical cancer clearer and give some useful suggestions for clinical application.

Introduction

Cervical cancer is the second leading cause of cancer morbidity and mortality for women around the world, especially in many developing countries. In 2005, there were, according to WHO projections, >500,000 new cases of cervical cancer, of which >90% were in developing countries. It is estimated that over one million women worldwide currently have cervical cancer (1,2), most of whom have no access to treatment that could cure them or prolong their life. At present the demographics of cervical cancer patients are moving towards youngsters (3), majority of this population are single or nullipara women. As a result there exists a strong demand for an effective medical treatment to cure the cancer which preserves a women's fertility function. An effective therapy (4) technique is also required for those patients who are elderly, who cannot undergo surgery or radiotherapy, and those who refused surgery, or conservative treatment.

The genesis of photodynamic therapy in reproductive system started in the mid-1970s, with the first application to cure genital herpes (5). People attempted to treat cervical lesions with PDT in the late 1980s. PDT (6), as a conservative therapy, could reserve complete organic structure for the patients, and could be repeated multiple times, if necessary, to eradicate survival lesions. Overall, the efficacy and low risk of side-effects afforded by this therapy have resulted in high patient preference in clinical trials (7).

However, ALA-PDT is still immature in treating cervical intraepithelial neoplasia and early-invasive carcinoma of the cervix (8). The fundamental scientific research is largely lagging behind, compared to the development and application of photosensitizer and laser devices. Current research could not provide a quantitative description of the process of photodynamic reaction, and interactions among the photodynamic factors. Clinicians could only treat the various patients by following the same pattern, which was built based on old experiences in the 1980s (9).

Through in vivo and in vitro experiments, our study searches for the optimal condition and accurate parameters of PDT, to analyze the mechanism of cervical cancer cell death after PDT. By comparing the effects of various modes of administration, our study identifies the optimal mode to reduce phototoxic reaction brought by PDT. The results of
the study provide valuable references in standardizing and quantifying PDT clinical applications for millions of patients.

Materials and methods

Cell culture. The Me180 cell line was given by Dr Takeshi Yamashita from Asahikawa Medical College in Japan. The cells were routinely propagated in monolayer cultures in Dulbecco’s modified Eagle’s medium (Gibco-BRL, Paisley, Scotland), supplemented with 5% heat-inactivated fetal bovine serum, 0.37% sodium bicarbonate, 30 mM HEPES, and penicillin/streptomycin. The cells were cultured in a 5% CO2 incubator at 37°C. The cells were serially subcultured normally and the cells at the logarithmic growth phase were chosen for experiments.

Chemicals. ALA obtained from Aldrich and Sigma Chemical (St. Louis, MO, USA), was dissolved in PBS and PH was adjusted to 7.0 by addition of 5 M of NaOH. The stock solutions of 50 mmol/l were made and kept at -20°C before use. All of the other chemicals used were of the highest purity commercially available.

Animals. Female BALB/c nude mice were obtained from Vitalriver Co. (Beijing, P.R. China) and maintained under specific pathogen-free conditions. The mice were 6-8 weeks old and weighed 20-25 g when the experiments started.

In vitro and in vivo treatment protocols. ALA was dissolved directly before injections. The cells under study were incubated with ALA (prepared by dissolving freeze-dried ALA in a medium) at various concentration (10, 1, 0.1, 0.01, 0.001, 0.0001 mmol/l, respectively) in serum-free medium and incubated for 4 h. The exponentially growing cells were washed in PBS 3 times before fresh medium was added. The PDT was carried out using a He-Ne laser generator apparatus (QingHua University Laser Technology Company, P.R. China). The wavelength was set at 632.8 nm. The cultures were then subjected to laser irradiation (10, 20, 30 J/cm2, respectively), and then returned to the incubator. The cell inhibitive rate was determined by MTT assay. After the addition of photosensitizer to the cells, all procedures were carried out in minimal ambient lighting.

Animals were randomized into one of six groups (12 animals in each group): pure topical administration group (undergoing none of the treatment), pure intravenous administration group (undergoing injection of ALA 250 mg/kg into the caudal vein), pure radiotherapy group (undergoing radiotherapy without ALA), topical administration and radiotherapy group (undergoing injection of ALA 250 mg/kg into the caudal vein and radiotherapy of 630-nm He-Ne laser 3 h after the drug administration), intravenous administration and PDT group (undergoing injection of ALA 60 mg/kg around the tumor and radiotherapy of 630-nm He-Ne laser 3 h after the drug administration), intravenous administration and PDT group (undergoing injection of ALA 250 mg/kg into the caudal vein and radiotherapy of 630-nm He-Ne laser 3 h after the drug administration) and control group (undergoing none of the treatment). The animals under study were treated with freeze-dried ALA dissolved in physiologic saline. The time intervals between drug administration and light irradiation were chosen as 3 h. This was on the basis of a previous study (10).

### MTT assay

The Me180 cell lines were inoculated into an 96-well plate at a volume of 200 μl (5x10^4 cells/well) for stationary culture. Twenty-four hours later, different concentration of ALA-PDT was carried out using a He-Ne laser generator apparatus (QingHua University Laser Technology Company, P.R. China). The wavelength was set at 632.8 nm. The cultures were then subjected to laser irradiation (10, 20, 30 J/cm^2). The laser radiated the single layer of cells from up to down. After radiation, DMEM was replaced with fresh DMEM medium and the cells were cultured for 1, 2, 3, 6, 12, and 24 h with avoidance of light. Then, for the MTT assay, 20 μl of 5X MTT was added to each cell-culture well and cultured for 3 h. The absorbance was measured with a microplate reader (Multiskan MS, Labsystem, Finland) at 562 nm. Measurements were performed at 1, 2, 3, 6, 12, and 24 h after laser irradiation to screen the optimal function time of ALA-PDT. Each group consisted of four wells; the means of their values were used as the measured values. The cell inhibitive rate was calculated according to the formula: Cell inhibitive rate (%) = [(OD values of control cells - OD values of treated cells)/OD values of control cells] x 100%. The time course of the changes in inhibitive rate after laser irradiation was observed.

Morphologic changes induced by PDT. After the ALA-PDT functioned for 3 h, cytospin slides were gathered and cell climbing slides were prepared in the same manner. Then the cells were fixed with methanol. The cell nuclei were stained by May-Grünwald-Garnstoff for 4 min (2.5 g per 1000 ml) and then were cleansed with distilled water. Giemsa (0.5 g glycerc per 33 ml for 2 h at 56°C and then 33 ml methanol added) was used to counterstain the cytoplasm for 15 min. Then the cells were cleansed with tap water. The cells were treated with xylene, and neutral resin
was used to seal the slides. The cell morphology was observed under a microscope.

After PDT treatment, the cells were incubated in the dark with Hoechst 33342 (Beyotime Biotechnology, P.R. China) for 30 min at 37°C and were then observed with a fluorescence microscope to detect morphologic changes in the nuclear chromatin of cells undergoing apoptosis compared with control.

Apoptotic analysis using dual Annexin V-FITC/propidium iodide. The Me180 cells were inoculated in the 6-well plate and were cultured for 24 h. After the medium was replaced with the fresh, ALA was added to maintain the final concentration of 0.1, 1 and 2 mmol/l. In the meantime, the blank control group was set up. At 6 h after ALA was added, the radiation energy of 20 J/cm² was adopted for radiation. Then the DMEM was replaced with fresh medium and the cells continued to be cultured for 24 h with avoidance of light. The cells were gathered and cleansed by PBS twice. Cell suspensions were centrifuged and resuspended in phosphate-buffered saline (PBS) to a concentration of 10⁶ cells/ml. For flow cytometric analysis, cells were incubated with 5 μl of Annexin V-fluorescein isothiocyanate (BD, Pharmingen) and 10 μl of propidium iodide (PI) in the dark at room temperature for 10 min followed by fixation with 2% formaldehyde. The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS) in a FACScan (Beckman Coulter Epics XL, USA). The forward- and side-scatter gates were set to exclude any dead cells from the analysis; at least 10,000 events were collected for each sample. The observations were performed at 3 and 24 h after PDT.

Cell cycle analysis using propidium iodide. The culture medium was removed and saved after the ALA-PDT functioned for 24 h, cells were trypsinized and returned to the medium they had grown in and then centrifuged. The cells were then washed twice in ice-cold phosphate-buffered saline (PBS) and stored at -20°C in 80% ethanol until required. Fixed cells were resuspended in 50 μl RANse (0.1 U/ml) and 300 μl of the stoichiometric dye propidium iodide (50 μg/ml). Cells were incubated at 4°C overnight prior to analysis with FACScan. At least 10,000 events were collected for each sample. DNA histograms were analyzed using EXPO32 ADC software.

Real-time reverse transcription-PCR. The cells were gathered after the ALA-PDT functioned for 3 h. RNeasy® RNA extraction kit (Qiagen) was used to extract the total RNA and the ultraviolet spectrophotometer to measure the concentration of RNA. The RT-PCR continued according to the instructions given in the reverse transcriptase kit from MBI company.

Each cDNA sample was analyzed in triplicate (aliquot of 1 μl each) using the ABI PRISM 7300 sequence detector (PE Applied Biosystems, Germany). Quantitative assessment of DNA amplification was detected through SYBR Green dye. The RQ-PCR reactions were carried out in a total volume of 50 μl according to the manufacturer’s manual for SYBR Green PCR Core reagent (Takara Biotechnology Inc., P.R. China). The sequence detector software 1.7 calculates the threshold cycle number (Ct) when signals reach 10 times the SD of the baseline. It was previously demonstrated that the calculated Ct values are a quantitative measurement for the mRNA levels of various genes tested. RT reaction (2.5 μl), or water as control, was amplified in triplicate by real-time PCR in a final volume of 50 μl using the SYBR Green Master mix reagent at a final concentration of 1X (Takara Biotechnology Inc.). The primer pairs (11-13) for detecting the expression of genes are listed in Table I.

For thermal cycling, the following conditions were applied: 2 initial incubations of 2 min at 50°C and 10 sec at 95°C, then 45 cycles of 5 sec at 95°C and 1 min at 60°C. The heating rate (ramping) between the last 2 steps was increased to 20 min to obtain a melting curve of the final RQ-PCR products (PE Applied Biosystems). This is necessary because SYBR Green fluorescence may also be derived from side products such as primer-dimers. The ΔCt values were calculated according to the following formula: ΔCt(target gene of PDT group) = Ct(target gene of control group) - Ctß-actin of the same sample; ΔCt(target gene of control group) = Ct(target gene of control group) - Ctß-actin of the same sample; ΔCt between different groups was compared.

Inhibition of tumor growth. The responses of treatments were evaluated by measuring survival rates and tumor size. The following protocol of experiments was used. A 0.1 ml of PBS suspension (5x10⁶ cells/ml) of Me180 cells was injected subcutaneously with a syringe into the flank of mice. At this site the tumor was easily accessible to treatment and to assessment of response. After the cancer cells were allowed to grow to a certain level, ALA was injected of each mouse of the PDT group. The photodynamic treatment was carried out 24 h after the drug administration using 630-nm radiation of a He-Ne laser. The tumor volume was calculated using the following formula: V=π/6(D₁xD₂xD₃), where D₁, D₂ and D₃ were three orthogonal diameters of the tumors that were evaluated once every 2 days for 3 weeks using calipers, and tumor size calculated based on average dimensions. No spontaneous necrosis was observed in the tumors on the day of treatment. The tumors were resected on the days indicated and stored at -70°C.

Histopathologic examination. After measuring the gross tumor size, the specimens were embedded in paraffin, cut into 5-μm slices, and mounted on slides. The sample was stained with hematoxylin-eosin and examined under a light microscope.

Statistical analysis. The data were calculated and analyzed via the Excel and SPSS 10.0 package. Statistical analysis was done using the paired Student’s t-test and ANOVA. Values between different groups were compared. P<0.05 was considered significant.

Results

Cell growth-suppression activity of PDT. ALA-PDT does have the effects of growth inhibition on all cervical cancer lines in vitro, the phototoxic IC₅₀ concentration in Me180 is 7.28x10⁻⁴ mM. Fig. 1 shows the concentrations of ALA that give rise to 50% (IC₅₀), 75% (IC₇₅), and 90% (IC₉₀) growth inhibition of Me180 cells. From 1x10⁻⁴ to 1x10⁻¹ mmol/l, the proliferation inhibition rate increased regarding the increase
of the drug concentration (P<0.01). From 0.1 to 10 mmol/l, the cell proliferation inhibition rate did not increase obviously. There is no obvious phototoxic reaction at <10 mM and 30 J/cm².

The IC₅₀ was (9.99±0.24)x10⁻³ mmol/l 1 h post-PDT. It increased gradually at 2 and 3 h, and reaches its peaks at 4 h post-PDT that IC₅₀ was (5.05±0.22)x10⁻⁴ mmol/l. There is significantly difference comparing to 1, 2 and 3 h. The IC₅₀ decreased at 6, 12, 24 h post-PDT. The IC₅₀ at 24 h was (1.45±0.24)x10⁻³ mmol/l. The optimal results come at 4 h after ALA-PDT.

**Figure 1.** Cell growth-suppression effect of PDT. Cervical cancer cell Me180 was grown in different concentration of ALA (10⁻⁶-10 mmol/l) and different laser irradiation (10, 20, 30 J/cm²). Also the inhibition of cells was determined by the MTT assay.

**Figure 3.** Effects of ALA-PDT on apoptosis of Me180 cells (Hoechst 33342, x200). (A) Only a few apoptotic cells can be seen in control group; (B) Certain swollen cells can be seen after treatment of 0.1 mmol/l ALA. (C) Apoptotic cells and apoptotic bodies can be seen after treatment of 1 mmol/l ALA. (D) More apoptotic cells and apoptotic bodies, swollen cells and cell death can be seen after treatment of 2 mmol/l ALA.

**Table II.** Effects of ALA-PDT on cell cycle of Me180 cells at 24 h.

<table>
<thead>
<tr>
<th>Group</th>
<th>G0-G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.64±3.23</td>
<td>29.25±4.34</td>
<td>28.97±4.78</td>
</tr>
<tr>
<td>ALA-PDT (0.1 mmol/l)</td>
<td>45.28±5.54</td>
<td>27.31±5.43</td>
<td>28.26±5.34</td>
</tr>
<tr>
<td>ALA-PDT (1 mmol/l)</td>
<td>61.06±6.97⁺</td>
<td>20.12±3.12⁺</td>
<td>18.83±4.39⁺</td>
</tr>
<tr>
<td>ALA-PDT (2 mmol/l)</td>
<td>69.58±5.12⁻</td>
<td>14.21±2.14⁻</td>
<td>16.11±3.47⁻</td>
</tr>
</tbody>
</table>

All values are presented as mean ± SD of 3 experiments. ⁺P<0.05, ⁺⁺P<0.01, ⁺⁺⁺P<0.001, vs. control.

**Morphologic changes after PDT.** We detected abundant amounts of apoptotic cells and some necrotic cells both by May-Grunwald-Farbstoff Giemsa staining (Fig. 2) and Hoechst 33342 staining (Fig. 3). Cell apoptosis was observed by monitoring cellular shape, May-Grunwald Giemsa staining, Hoechst 33342 and DNA content analysis using flow cytometry.

**DNA cell cycle analysis.** No significant cell cycle effects were evident at 4 h post-PDT, and the distribution of cell cycle significantly changed at 24 h post-PDT; an appreciable accumulation of cells residing in G0/G1 phase was induced at 1-2 mM, the reverse was seen in the S and G2/M phase (Table II).

**Cell death analysis.** Me180 cell death is significantly induced post ALA-PDT, in which the pattern of early apoptosis shows...
The degree of apoptosis was assessed 3 h and 24 h post-PDT as determined by flow cytometric analysis of the Annexin-FITC/PI double staining method; "P<0.01.

Figure 4. The degree of apoptosis was assessed 3 h and 24 h post-PDT as determined by flow cytometric analysis of the Annexin-FITC/PI double staining method; "P<0.01.

Figure 4. The degree of apoptosis was assessed 3 h and 24 h post-PDT as determined by flow cytometric analysis of the Annexin-FITC/PI double staining method; "P<0.01.

at 3 h post-PDT, and the total rate of apoptosis declines in 24 h post-PDT (Fig. 4), which could be considered as a different phase issue. The total rate of apoptosis for 1 and 2 mM are 52.45 and 77.92% respectively, 3 h post-PDT.

Real-time quantitative RT-PCR analysis in vitro. Results representative of real-time quantitative PCR are shown in Fig. 5 for bcl-2, survivin, p53, bax and bad. The ΔCt value of bcl-2 and survivin post-PDT is higher than control cells, which is reflective of a lower mRNA expression for bcl-2 and survivin (P<0.001). We observed that the ΔCt value of p53, bax and bad had only increased slightly (P>0.05).

Discussion

Different optimal PDT parameters have been reported (14-16). That could be caused by various types of cells, photosensitizers, and wavelength of laser chosen by different research entities. We found that ALA-PDT can inhibit the growth of cervical cancer cells in vitro. PDT is effective (17-19). After treatment with concentration <10^-1 mM, the cell survival rate decreased sharply. It shows a slower decrease when the concentration is >10^-1 mM, thereafter reaching a plateau. In ALA-PDT, 5-ALA itself does not serve as photosensitizer but as the biological precursor in the heme biosynthetic pathway (12,20), which produces protoporphyrin IX (PpIX), a potent photosensitizer, in response to 5-ALA-induced endogenous photosensitization. Cellular PpIX production increased, as suggested by our findings, after treatment of the cells with 5-ALA at relatively lower concentrations, and this increment exhibited saturation with higher concentrations of 5-ALA. As PpIX is the product in the heme biosynthetic pathway whose biosynthetic capacity is limited (21), the saturation of cellular PpIX production at higher 5-ALA concentration is not surprising. This finding may potentially help in deciding the optimal dose of 5-ALA in future clinical PDT.

The Annexin-V/PI technique has previously been employed in PDT studies and many types of mammalian cells undergo apoptosis after exposure to PDT (22,23). The cell death increased significantly after 1 h of PDT, and reached its peaks at 4 h and decreased gradually since 6 h. Also the cell re-growth tendency appeared after 24 h post-PDT. We speculate that the cell proliferation counteracted part of lethal
The perturbation of cell cycle appeared at 24 h post-PDT. An appreciable accumulation of cells residing in G0/G1 phase was induced at 1-2 mM, the reverse results were seen in the S and G2/M phase, as described (24). Almost all cells were destroyed when ALA 2 mM, effective light dose 10 J/cm² and 4 h post-PDT, which are the most optimal parameters.

The main purpose of *in vivo* study is to find a better mode of this drug so that phototoxicity of normal tissues could be avoided, while the effects retained. Tumor inhibition experiments *in vivo* demonstrated that there is no significant difference in therapy effects between the inhibition rate of intravenous (89.79%) and topical (85.44%) application. However, comparing the intravenous administration and the topical application, the topical application brings lower systemic uptake. Therefore, the topical administration is better.
mode (25) to reduce the phototoxic reaction in the PDT for cervical cancer.

The tumor volume was significantly reduced post-PDT by topical and caudal vein administration comparing to control group (P<0.001). In which the most significant effect of tumor size reduced the most, observed at topical treatment 7-14 days post-PDT. Our data are in agreement with the result of Ahn et al (26). It is helpful in deciding the optimal timing of the second PDT treatment. After 14 days, the size of tumor starts to increase again, which proves that the inhibitive time of single time PDT is un-abiding. The cause could be that the penetration depth of laser is limited, and the basal part of tumor keeps growing while the superficial layer necrosed. To improve the clinical effects, we suggest providing multiple periods of interrupted treatment. Also histologic analysis showed that PDT-treated tumors demonstrated necrosis and inflammation that was not seen in the control.

Previous studies have demonstrated complexity of cell death induced by PDT between various cell types in intra-cellular accumulation and phototoxicity of photosensitizer (27-29). It is reasonable to suppose that different photosensitizers, laser dose acting on different type of target cells would lead to different results. Those mechanisms are unclear (30-32). Various studies from in vitro culture systems and in vivo animal models have been proposed to explain the mechanism of both necrosis and apoptosis of the target cells by PDT (33,34). In this study, we evaluated the mRNA changes of related apoptosis genes. The in vivo and in vitro mRNA expression of survivin and bcl-2 was down-regulated in the experimental groups versus the control group (P<0.01), the mRNA expression of p53, bax, and bcl-2 was up-regulated in the experimental groups (P>0.05). It suggests that the cell death and necrosis of tumor tissue after PDT has high correlation with survivin and bcl-2. Our results might help in formulating new therapeutic approaches in photodynamic therapy. Future studies could be designed to document the precise pathway to apoptosis in cervical cancer, enhancing the induction of apoptosis.

In conclusion, the analyzing experiments in vivo and in vitro, indicate that ALA-PDT is an effective therapy for cervical cancer. We found that ALA-PDT exerts its anti-tumor activity primarily by inducing apoptosis. As such, we infer that early induction of apoptosis of Me180 is one of the important mechanisms in vitro and in vivo. The observations may be useful in understanding the apoptosis pathway by PDT. Topical administration of PDT is recommended in treating cervical cancer so as to minimize the side-effects and inconvenience of phototoxic reaction brought on by PDT.

References


