Photogem Induces Necrosis in Various Uterine Cervical
cancer Cell Lines by PDT

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Purpose: In order to elucidate the antitumor effect of photodynamic therapy (PDT), using a derivative of the photosensitizing agent hematoporphyrin (Photogem) and a diode laser, the cell death of uterine cancer cell lines (CaSki, HT3, HeLa, and SKOV-3), and mice transplanted with TC-1 lung cancer cells, were evaluated.

Materials and Methods: The morphological changes, MTT assay, flow cytometry, cytotoxicity and tumor growth inhibition study were evaluated at various time intervals after the PDT.

Results: The results showed that the survival rates of each cell line decreased with time and dose response after performing the PDT. Also, the PDT-induced damage of cancer cells was almost entirely confined to necrosis of the tumor cells in the early time courses. The irradiation of CaSki cells in the presence of Photogem induced plasma membrane disruption and cell shrinkage, indicating the plasma membrane as the main target for Photogem. In the in vivo experiment, significantly longer survival and a significantly smaller tumor size were seen over the time courses of the Photogem with irradiation compared to the untreated control groups; resorption of the tumor was also observed after the PDT treatment.

Conclusion: Collectively, our results indicated that Photogem possesses anti-tumor effects, and necrosis-like death, with plasma membrane damage, was postulated to be the principal mechanism of the antitumor effect of the PDT using Photogem. (Cancer Research and Treatment 2003;35:549-556)

Key Words: Photodynamic therapy, Photogem, Cervical neoplasm

INTRODUCTION

Photodynamic therapy (PDT) is principally a new method of treating malignant tumors, based on the use of photodynamical damage of tumor cells under a photochemical reaction (1 ~ 3). During the last several years, a whole range of dyes, such as Photofrin (Axcian Scandinpharm Inc., Birmingham, AL), HPD (Beijing Institute of Pharmaceutical, Beijjing, China), Photogem (TimTec Corp., Newark, DE), Benzoporphyrin derivative (QLT Inc., Vancouver, Canada), 5-aminolevulenic acid (PhotoCure Inc., Oslo, Norway) and others, have been used as photosensitizers for a wide range of malignant tumors, as well as non-malignant diseases (4 ~ 6). A tumor treated by PDT was resolved, and gradually substituted by connecting tissues. The locality of photodynamic damage of a tumor is provided by the ability of a photosensitizer to accumulate in tumor tissues, with the help of directed, localized, precise laser irradiation (7 ~ 9).

Photogem has demonstrated a considerable photodynamic effect (10,11). At present, PDT, using a hematoporphyrin derivative type photosensitizer, Photogem, is used clinically in the field of gynecology for the treatment of dysplasia and carcinomas, in situ, of the uterine cervix (12). Nowadays, Photogem is the officially approved photosensitizer for PDT of cancer in Russia. More than 1,500 patients have completed a course of treatment with Photogem (13). Positive effects from this method of treatment were observed in 99.4% of patients (total resorption of tumors was 56.2% and partial resorption 38.2%). Photogem transfers to an excited state in the affected cells, generating a complex of photodynamic and photochemical reactions, which lead to the destruction of cancerous cells. Another clinical use of this therapy aims to determine the tumor boundaries before the photodynamic treatment (14). 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, but little is known about the kinetics of the intracellular process after the

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PDT (15 ~ 17). To clarify the anti-tumor effects underlying the activity of cell death, both in vitro and in vivo, using cervical carcinoma cell lines, an experiment was performed, which showed that plasma membranes are the main target of Photogem. It was found that PDT, using Photogem, exerts its antimut activity primarily through inducing necrosis, rather than by inducing apoptosis.

### MATERIALS AND METHODS

1) **Cell cultures**

The cell lines tested included: CaSki, HT3 (derived from human cervical SCC), HeLa (derived from human cervical adenocarcinoma), SKOV-3 (derived from human ovarian cancer cells) and TC-1 (derived from murine lung cancer cells). The cell lines were obtained from the cell line bank at Seoul National University’s Cancer Research Center (Korea). The cells were routinely propagated in monolayer cultures in DMEM (GIBCO-BRL, Paisley, Scotland), supplemented with 5% heat-inactivated fetal bovine serum, 0.37% sodium bicarbonate, 30mM HEPES and penicillin/streptomycin. The cells were cultured in a 5% CO2 incubator at 37°C. Unless otherwise specified, all chemicals were purchased from Aldrich and Sigma Chemical (St. Louis, MO).

2) **Animals**

The female C57BL/6 mice (6 ~ 8 weeks old) were purchased from DaeHan BioLink (Daejeon, Korea), and maintained under pathogen-free conditions.

3) **PDT**

Photogem (TimTec Corp., Newark, DE) is a mixture of monomers and oligomers of hematoporphyrin derivatives (A=630-nm), and was dissolved directly before injections. To receive the active solution it was necessary to administrate 40ml of a physiological solution into a bottle with the powder. The bottle was shaken, and kept for 3 ~ 5 min, until the foam disappeared. The cells in the study were exposed to a solution of Photogem (prepared by dissolving freeze-dried Photogem in a medium). The animals in the study were treated with freeze-dried Photogem dissolved in physiological saline. The PDT was carried out using a diode laser generator apparatus (Biolitec AG, Jena, Germany), equipped with a halogen lamp, a band-pass filter (600 ~ 1,000-nm) and a fiber optics bundle. The wavelength was set at 630±1-nm. The duration of the light irradiation, under the PDT treatment, was calculated taking into account the empirically found effective dose of light energy in J/cm².

4) **Morphologic changes induced by PDT**

Cells of each of the cell lines were inoculated into an eight-well chamber slide, in a volume of 100µl (5×10⁵ cells/well) for the stationary cultures. Twenty-four hours later, Photogem (2.5µg/ml) was added at a volume of 100µl/well. After a predetermined time, the Photogem solution was discarded, and the culture was washed three times in physiological saline, and then medium added at a volume of 100µl/well. The cultures were then subjected to laser irradiation (2 J/cm²). After predetermined times, optical microscopy was used to determine the morphological changes induced, and compared with those in the cultures not subjected to laser irradiation. The time course of the changes in the survival rate after laser irradiation was observed. Also, the cells were examined in a JEOL 100/CX electron microscope.

5) **MTT assay**

Cells of each cell line were inoculated into a 96-well, flat-bottomed microplate, at a volume of 100µl (1×10⁵ cells/well), for the stationary culture. Twenty-four hours later, the medium was removed, and the cultures washed three times in physiological saline. Either 1.25, 2.5 or 5µg of Photogem was then added, at a volume of 100µl/well. Twenty-four hours later, the Photogem solution was discarded, the cultures washed again, three times in physiological saline, and medium added at a volume of 100µl/well. The cultures were then subjected to laser irradiation (2 J/cm²), followed by the MTT assay to evaluate their sensitivity to the PDT. For the MTT assay, 20µl of 5x MTT was added to each cell culture well, and cultured for 3 h. 200µl of DMSO was added to the culture, shaken for 10 min and the absorbance measured at 570-nm with an ELISA-reader. Measurements were performed 24 and 48 hours after the laser irradiation. Each group consisted of four wells; their mean values were used as the measured values. The time course of the changes in the survival rate after laser irradiation was observed. The amount of Photogem was also compared between the cancer cell lines.

6) **FACS analysis**

Cell suspensions were centrifuged and resuspended in phosphate-buffered saline (PBS) to a concentration of 10⁶ cells/ml. For flow cytometric analysis, the cells were incubated with 5µl Annexin V-FITC and 10µl propidium iodide (PI) in a dark, at room temperature, for 10min, followed by fixation with 2% formaldehyde (18). The stained cells were analyzed for DNA content by fluorescence-activated cell sorting (FACS), in a FACScan (Becton Dickinson, San Jose, CA). 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 1, 3, 6, 12, 24 and 48 hours after the PDT. CaSki cells that received neither Photogem nor laser irradiation served as the controls.

7) **Inhibition of tumor growth**

The animals were randomized into one of four groups (ten animals in each group): I, control, untreated; II, Photogem only; III, irradiation only and IV, Photogem and irradiation treatment. The responses to the treatments were evaluated by measuring the survival rates and tumor sizes. The following protocol for the experiments was used: A 0.1 ml PBS suspension (3×10⁵ cells/ml) of TC-1 cells was subcutaneously injected, with a syringe, into the belly of the transgenic mice. After the cancer cells had grown to a certain level, the Photogem was injected, via the tail vein, of the PDT group, at a dose of 5 mg/kg body weights. The photodynamic treatment was carried out 24 h after the drug administration, using 630-nm radiation with a diode laser. A power density of 400 mW/cm² and irradiation time of 250 sec
was used. The tumor formation and size were evaluated once every two days, for 4 weeks, by measuring two perpendicular diameters, with calipers, and the tumor sizes calculated based on average dimensions. The tumors were resected on the days indicated, frozen to -70°C and stored until required for analysis.

8) Statistical analysis

The statistical analyses were performed using the paired Student’s t test and ANOVA. Values between different groups were compared, and p values < 0.05 were considered significant.

RESULTS

1) Morphologic changes after PDT

Fig. 1 shows optical and transmission electron micrographs illustrating the morphological changes of the CaSki cells at different time points after the PDT. The untreated CaSki cells,

![Figure 1](image1)

**Fig. 1.** Development of Photogem-mediated morphological changes in CaSki cells. PDT treatment (200 mW/cm², 10 sec), with Photogem 2.5μg/ml, followed by 3, 6, 12, 24 and 48 hr incubation, showed morphological changes. Typical light micrographs are shown. The untreated CaSki cells and controls with Photogem alone show no significant morphological changes. ×400 for all photographs.

![Figure 2](image2)

**Fig. 2.** Representative transmission electron micrograph of CaSki cells under the same condition. The patterns of cell death shows the plasma membrane is the target of Photogem, followed by a yamlike processes formed around the cytoplasm, disintegrated cytoplasm, pronounced vacuolization, shrunken condensed cytosol, nuclear chromatin condensations, lysis, plasma membrane blebbing and extensive fragmentation of the nucleus, which confirm the loss of cell viability (TEM, magnification ×3,000).
and the control, with Photogem alone, showed no significant morphological changes. There were drastic changes in the state and activity of the cellular organelles after 1hr of the PDT. The PDT treatment against the CaSki cells induced plasma membrane disruption and cell shrinkage, indicating the plasma membrane was the main target for the Photogem. That is, the cell membranes of the CaSki cells began to shrink immediately after the PDT, and cell death processes, with cytoplasm leakage, formed around the membrane for 1hr, as shown in Fig. 2, but the morphology of the nuclear membranes did not alter significantly. Six hours later, the membranes disintegrated, confirming the loss of cell viability. Similar morphology changes were also obtained with the other tumor cell lines.

2) Cell growth suppression activity of PDT

The cell growth inhibition effect of the PDT, with Photogem, on the CaSki, HT3, HeLa and SKOV-3 was evaluated using MTT assays with increasing amounts of Photogem, and showed a significant increase in the cell growth inhibition over time. As shown in Fig. 3, the survival rate decreased, via the differential role of Photogem dose-specific cell death, compared to untreated control cells, which was cancer cell line dependent. The growth inhibition of the SKOV-3 and HT3 cells was clearly shown to be Photogem dose-dependent at 48 hr post treatment. In contrast, the CaSki cells showed growth inhibition patterns at all Photogem doses, but did not show the dose-dependent effect at 48 hr post PDT. The growth inhibition of the HeLa cells plateaued at 48 hr post PDT, an showed no difference (Photogem-resistant) at 24 hr post PDT at any Photogem dose, with a little dose response only at 5μg/ml thereafter. Thus, the growth inhibition of each cell line, at 48 hr post PDT with any Photogem dose, was shown to be cell type-dependent, compared to each control group. At 24 hr post PDT, only the HT3 cells showed Photogem dose effects. That is, the growth inhibition levels of the HT3 cells increased sharply at 5μg/ml, 24 hr post PDT. In contrast, the CaSki cells showed a growth inhibition response only at 5μg/ml of PDT. The growth inhibition levels at 24 hr post PDT were signi-
stantly higher for the CaSki and HT3 cells than for the SKOV-3 cells. Taken together, the MTT assays showed the differential relationship between the post PDT treatment time and the PDT dose. Also, the growth inhibition, post Photogem-PDT, was cancer cell line dependent.

3) Cell death induced by PDT

The PDT has been shown to mediate cell death in several cell types. The uptake of Photogem prevents cells from undergoing proliferation after PDT, and increases the efficacy

![Flow cytometric analysis of apoptotic cells](image_url)

**Fig. 4.** Inductions of apoptosis and necrosis by the PDT. Quantitative analysis of the apoptotic cells, using annexin-V FITC and PI, in exponentially growing CaSki cells (A), control (B), control of Photogem alone and (C) control of laser alone. In the case of the PDT treatment (200 mW/cm², 10 sec), with Photogem 2.5μg/ml, the CaSki cells were incubated for 1, 3, 6, 12, 24 and 48 hr. The flow cytometric analysis was performed on 10,000 cells, and the percentages of apoptotic (A), live (L) and dead (D) cells were measured. The left lower quadrant represents live cells, the right lower quadrant apoptotic cells and the top right quadrant dead cells. This experiment was repeated, with similar results, on at least three occasions.
of the antitumor effect, both in vitro and in vivo. To check whether the cell death was accompanied with the development of an apoptotic or necrotic process, the phenotypic changes characteristic of apoptotic cells were analyzed and quantified by double staining CaSkI cells with annexin V and propidium iodide (PI). Exponentially growing control cells were exposed to PDT, and then harvested at predetermined times. Fig. 4 shows that the cell death significantly and immediately increased post PDT, and the pattern of cell deaths at 1 and 3 h after PDT were almost the same levels as those observed with the morphology changes. That is, a greater induction of cell death as a consequence of Photogem uptake was observed for 1 h. The cell death of the CaSkI cells increased sharply during the first time period, but showed a slower decrease thereafter, reaching a plateau after about 12 hours of PDT. In the MTT assays, most cells showed a regrowth tendency after 24 h. A few cells underwent apoptosis (lower right panel) for 48 h post PDT. In contrast, after the early irradiation times, most cells were classified as necrotic. As expected, the cells exhibited a much higher susceptibility to necrosis in response to PDT, rather than early apoptosis. On the other hand, there was no detectable cell death effect with the control of Photogem alone. In further support of the cooperative action between apoptosis and necrosis, it is apparent from our results that the strong staining of annexin V-positive cells by PI showed the disruption of intact plasma membranes in the CaSkI cells, leading to necrotic cell death.

4) Antitumor activity of PDT

The antitumor activity of Photogem indicates that the survival rate response, as well as to cell growth inhibition, were similar. Fig. 5A shows the survival curve for the C57BL/6 transgenic mice transplanted with TC-1 cells. In the control group, the 50% survival period was 12 days. In the PDT group, however, most mice were still alive at 30 days. For mice with the administration of Photogem alone, the 50% survival period in the control group was about 15 days, and the one for irradiation alone was 25 days. Thus, the survivals of the PDT treatment or laser irradiation alone groups were significantly greater than in the other groups.

Fig. 5B shows the time course of the changes in the tumor sizes in the C57BL/6 transgenic mice transplanted with TC-1 cells. A significant difference was confirmed on day 8, and a tendency to grow larger was observed on all days. In the control group, the tumor sizes increased almost linearly with time, until the end of the observation period. In the Photogem alone group, linear increases in tumor sizes, similar to those in the control group, were observed after the PDT, indicating no cytotoxicity. In the PDT treatment group, however, the tumor sizes decreased after the beginning of the PDT, reached a minimum for 8–14 days, followed by a slow increase until the end of the observation period. The laser irradiation alone group showed a slight tumor growth delay compared to the trend of the control group.

**DISCUSSION**

PDT, using Photogem, has shown complete regression of tumors verified morphologically in 62%, partial regression in 34% and limited response or absence of an effect in 4% of cases (19). In this study, the CaSkI cells were shown to contain Photogem, which was excited to emit white fluorescence under fluorescence microscopy (wavelength 650-nm). When observed microscopically, the plasma membrane was the major target of Photogem, but little Photogem uptake into the nuclei was visible (data not shown). That was because Photogem mainly localized in the plasma membranes; PDT caused dramatic morphology changes of the CaSkI cells, leading to necrotic cell death. A previous study demonstrated accumulation of the lipophilic photosensitizer, Photogem, in the nuclear membrane, cytoplasmic membrane, endoplasmic reticulum and mitochondria. The findings were supported in this study, where the excited fluorescence from the Photogem was mainly shown in the plasma membrane, but not the nucleus, under fluorescent microscopy, and the morphological changes induced by the
PDT were almost entirely confined to the plasma membrane. The results indicate that plasma membrane-localized Photogem induces damage, and the consequence of the release of growth factors into the extracellular matrix may serve as a cause of necrosis in the PDT-treated cancer cells. The results of the flow cytometry indicated that irradiation of cells loaded with Photogem induces necrosis.

Conversely, it has been suggested that in vivo studies have to indicate the importance of apoptosis versus necrosis in a tumor cure by PDT and in the role of cellular rescue responses (20). In the in vivo experiment of this study, PDT, using Photogem, was determined as exerting its antitumor activity primarily by inducing a physical change, and finally the destruction of the cellular membranes of the tumor cells, resulting in tumor necrosis. With respect to the molecular mechanisms of the PDT-induced photodamage, it seems that membrane lipid peroxidation plays a main role in the killing of cells (21). This is consistent with the observation that porphyrins, as hydrophobic sensitizers, tend to localize in the plasma membrane.

As previous studies have demonstrated the complexity of cell death induced by PDT, between various cell types in intracellular accumulation and photosensitizing of photosensitizer (22,23), it is reasonable to suppose that the in vivo efficiency of Photogem can also vary for different tumor types (24). In this study, the differential effectiveness of the therapy, against HeLa (a cervical adenocarcinoma cell line), CaSkI, HT3 (cervical Squamous cell carcinoma cell lines) and SKOV-3 (ovarian cancer cell lines) were evaluated. The subcellular localization of the photosensitizer has been shown to be a key factor in the outcome of PDT (25). The necrosis-like death, with plasma membrane damage, against HeLa cells was comparable to that against other carcinomas of the cervix, suggesting a satisfactory effectiveness against cervical adenocarcinomas, which is of great relevance in gynecological practice, due to the known resistance of this type of tumor to chemo- and radiotherapy.

CONCLUSION

The mechanisms involved in the death of human cervical cancer cells, triggered by PDT with Photogem, were characterized, and showed that PDT using Photogem exerts its antitumor activity, primarily through inducing necrosis, rather than by inducing apoptosis. This therapy might have a potent inflammatory response to necrotic cervical carcinoma cell lines induced by PDT, and therefore lead to tumor specific immunity, which promise a decisive role in the treatment of gynecological cancer, with reduced incidences of adverse effects.

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