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☐ Original Article ☐

Chlorophyll Derivatives
– A New Photosensitizer for Photodynamic Therapy of Cancer in Mice –

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The in vivo photosensitizing efficacy of chlorophyll derivatives (CpD), which had been developed as a new photosensitizer, was compared with that of hematoporphyrin derivatives (HpD). A murine tumor model implanted subcutaneously with S-180 cells on the abdomen was used. The CpD or HpD was administered by intratumoral injection, and light of appropriate wavelength was irradiated on the tumor areas for 10 minutes at 1h and 24h or 24h and 48h after the injection of photosensitizer. When CpD was injected, the early irradiation group (1h and 24h) showed a 100% tumor cure rate; however, the late irradiation group (24h and 48h) showed a 60% tumor cure rate ($p<0.01$). This showed that the early irradiation with light after injection of CpD was an important factor for obtaining better results. With HpD, there was no difference in tumor cure rate between early (1h and 24h, 80%) and late irradiation (24h and 48h, 80%) groups. Thus, in early irradiation groups, the tumor cure rate using CpD (100%) was superior to that of HpD (80%) ($p<0.05$). However, in late irradiation groups, the tumor cure rate using CpD (60%) was inferior to that of HpD (80%), but this difference was not statistically significant ($p>0.1$). Pathologic sections of these tumors were made before treatment and 48h and 3 weeks after treatment. These showed geographic necrosis at 48h after treatment and no viable tumor tissue at 3 weeks after treatment. Our results showed that CpD was as effective as HpD as a photosensitizer for in vivo photodynamic therapy.

Key Words: Photosensitizer, chlorophyll derivatives (CpD), hematoporphyrin derivatives (HpD), photodynamic therapy (PDT)

Photodynamic therapy (PDT) is a new modality of treating tumors by the combined use of locally or systemically administered photosensitizers and local application of light. The photodynamic effect of acridine compounds was first reported by Oscar Raab in 1900. As early as 1903, Tappenier and Jesionek utilized this process in the treatment of malignant disease when they treated skin cancers using topical eosin as the photosensitizer together with white light. Numerous other sensitizers have subsequently been used as photosensitizers, for example, tetracycline (Rall *et al.* 1957), berberine sulfate (Mellors *et al.* 1952), acridine orange (Tomson *et al.* 1974), fluorescein (Tomson *et al.* 1974), and various porphyrins (Bellin *et al.* 1961; Fowlks 1959). Hematoporphyrin derivatives (HpD), the photosensitizer that has generated greated interest, is a synthetic derivative of hemoglobin (Dorion and Gomer 1984).

Effective use of porphyrin photosensitizers for antitiumor therapy has been documented at several clinical centers and has been used on several thousand patients with generally encouraging results (Cortese and Kinsey 1982; Hayata and Dougherty 1983; Dahlman *et al.* 1983; McCaughan 1987). It has been suggested that porphyrins accumulate selectively in malignant tissue, thus causing these tumors to fluoresce (Gregorie *et al.* 1968). When irradiated with

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an appropriate wavelength of light, these photosensitizers are excited to the triplet state and are then capable of reacting directly with tissue components or undergoing interaction with molecular oxygen in order to produce cytotoxic species such as singlet oxygen and free radicals (Weishaupt 1976). Although the history of porphyrins and their role as a diagnostic and therapeutic modality is relatively recent, a wide variety of human tumors with varying histological types have been treated. Good results with PDT have been reported with cancers of the skin (Dougherty *et al.* 1978), female genital tract (McCaughan *et al.* 1985), lung (Hayata and Kato 1983), esophagus (McCaughan *et al.* 1984, 1985), bladder (Benson *et al.* 1982), eye (Bruce 1984), breast (Dougherty *et al.* 1979), and oropharynx (Wile *et al.* 1982).

In spite of the demonstrated efficacy of HpD, there has been an intensive search for new photosensitizers. In 1987, the Department of Microbiology of Yonsei University College of Medicine, Yonsei Cancer Center, and the Departments of Applied Chemistry and Chemistry of Ajou University were able to discover a new photosensitizer consisting of chlorophyll derivatives (CpD), which can be easily obtained from natural resources (Lee *et al.* 1989). From preliminary in vitro experiments which were conducted previously, we were able to discover that the wavelength applicable (670nm) to CpD was longer than that of HpD (630nm). Therefore, tissue penetration would be better using CpD rather than HpD, and superior cytotoxicity and higher cellular concentration were noted using CpD (Lee *et al.* 1989). In order to determine the in vivo effectiveness of CpD, we performed experiments using the murine tumor model in the following study.

MATERIALS AND METHODS

Chlorophyll Derivatives (CpD)

The Department of Microbiology of Yonsei University College of Medicine, Yonsei Cancer Center, and the Departments of Applied Chemistry and Chemistry of Ajou University cooperatively developed a new photosensitizer which was composed of chlorophyll derivaties. CpD were extracted from fecal specimens of silk worms as a source of chlorophyll. The agent was found to be chemicals with a pyrol ring structure without Mg^{++} inside of the ring. This agent is water soluble and is a deep green powder. It was dissolved in normal saline to 5mg/ml as a stock solution and was kept in the dark at −20°C until used.

Hematoporphyrin Derivatives (HpD)

HpD was prepared from hematoporphyrin dihydrochloride (Sigma, U.S.A.) as described by Lipson *et al.* (1961). Stock solutions were prepared by dissolving the acetylated porphyrin in water containing 0.1 M NaOH. After 1h of stirring, the solution was brought to pH 7.2 by the addition of 0.1 M HCl and was sterile-filtered through a 0.22 u Milipore filter. By use of phosphate buffered saline, solutions of 5 mg/ml of HpD were made and kept in the dark at −20°C until used.

Tumor cell line (S-180)

The S-180 tumor cell line, cloned from the original S-180 (ATCC, Rockville, MD., U.S.A.), has been maintained in Yonsei University College of Medicine for more than 5 years in McCoy's 5a medium (FlowLab., Australia) containing 10% fetal bovine sera (FBS, FlowLab, Australia). The cells have been routinely maintained both in vitro as well as in vivo within the inbred strain of ICR mice. Cultures were grown in glass (Corning Co., Pyrex Co., U.S.A.) and plastic (Coster, U.S.A.) flasks as necessitated by the specific experiment. Changes in the stem cell lines were frequently monitored by studying their karyologic patterns.

Animals and Tumor Formation

ICR mice weighing approximately 25g were randomly selected for these experiments. The mice were fed a commercial diet throughout the experiments. They were kept under natural light before injection of the photosensitizer and then they were placed in the dark. Approximately 5×10^6 cells were injected subcutaneously into the abdomen of the animals. The diameter of the tumor reached approximately 5 and 10mm, 7 and 14 days after tumor transplantation respectively.

Light Source

The light source used for this experiment was the beam of a Singer projector using a 300 W Sylivania lamp. The red filter was used for CpD and the yellow filter was used for HpD since the proper absorption wavelengths for CpD and HpD are 670nm and 630nm respectively.

Photodynamic Therapy

ICR mice with sarcoma on the abdomen were divided into 4 groups of 20 animals according to

agents used and treatment time intervals; groups A-1, A-2, B-1, and B-2. These mice were treated with light for 10 minutes each per treatment. During the 10 minutes, 60 Joules was irradiated into the tumor area.

A-1 group: 0.25mg of CpD was injected intratumorally and then PDT was performed for 10 minutes after 1h and 24h.

A-2 group: 0.25mg of CpD was injected intratumorally and then PDT was performed for 10 minutes after 24h and 48h.

B-1 group: 0.25mg of HpD was injected intratumorally and then PDT was performed for 10 minutes after 1h and 24h.

B-2 group: 0.26mg of HpD was injected intratumorally and then PDT was performed for 10 minutes after 24h and 48h.

Histologic Preparation

Tumors were cut with the surrounding abdominal tissue and fixed in 10% phosphate buffered formalin. The fixed tissue was embedded in paraffin, sectioned at 5 um intervals and stained with hematoxylin and eosin. The slides were then examined with an Olympus BH-2 microscope fitted with an Olympus C-35 AD-2 camera.

RESULTS

Cure is defined as no palpable tumor mass at least 6 weeks after treatment. After the treatment with CpD, all 20 mice in A-1 group showed complete remission (cure) and 12 of the 20 mice in A-2 group showed complete remission. The remaining 8 mice in A-2 group showed partial remission and tumor regrowth after 1 week. After the treatment with HpD, 16 of the 20 mice in B-1 group showed complete remission and 16 of the 20 mice in B-2 group showed complete remission (Table 1). The X^2 test was used as the statistical method. In using CpD, the tumor cure rate was superior in A-1 group (100%) compared to A-2 group (60%) ($p<0.01$). This showed that early treatment with light after injection of CpD was an important factor for obtaining good results. In using HpD, there was no difference in tumor cure rate between B-1 and B-2 groups. In the case of treatment after 1h and 24h, the response using CpD (A-1, 100%) was superior to that of HpD (B-1, 80%) ($p<0.05$). However, in the case of treatment after 24h and 48h, the response using CpD (A-2, 60%) was inferior to that of HpD (B-2, 80%), but it was not statistically significant ($p>0.1$).

During the treatment with CpD, necrosis of the

Table 1. Tumor response to different photosensitizers and time intervals for light irradiation

Photosensitizer	Group	Light irradiation (10 min). Post-injection	Number of Mice cured/total(%)
Chlorophyll	A-1	1h and 24h	20/20 (100%)
Derivatives (CpD)	A-2	24h and 48h	12/20 (60%)
Hematoporphyrin	B-1	1h and 24h	16/20 (80%)
Derivatives (HpD)	B-2	24h and 48h	16/20 (80%)

X^2 test: A-1 vs A-2: $p<0.01$
A-1 vs B-1: $p<0.05$
A-2 vs B-2: $p>0.1$

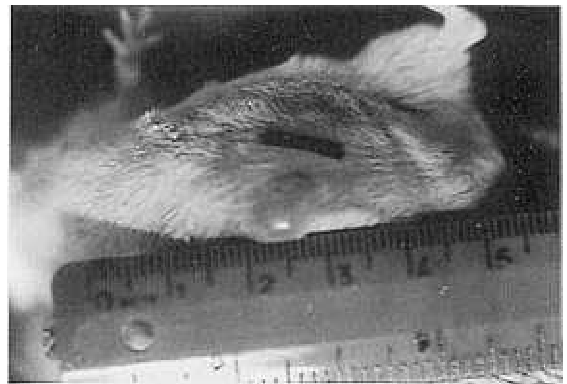

Fig. 1. Subcutaneous tumor mass in an ICR mouse implanted with S-180 cells (14 days after inoculation).

Fig. 2. Scab on the abdomen on 7th day after photodynamic therapy with CpD.

tumor became apparent as early as 1 day after the first light exposure and ultimately a scab formed over the tumor area. The entire tumor mass was generally reduced to a nonpalpable size within a few days after the first treatment. Eventually the skin completely healed and the hair usually regrew. This series of events is depicted in Figures 1-3.

In the experimental animals, there was complete disappearance of the palpable tumor mass with pathologically confirmed tumor necrosis beginning 24 hours after light treatment. The pathologic findings at 24h

Fig. 3. Completely healed skin and regrowing hair at 4 weeks after photodynamic therapy with CpD.

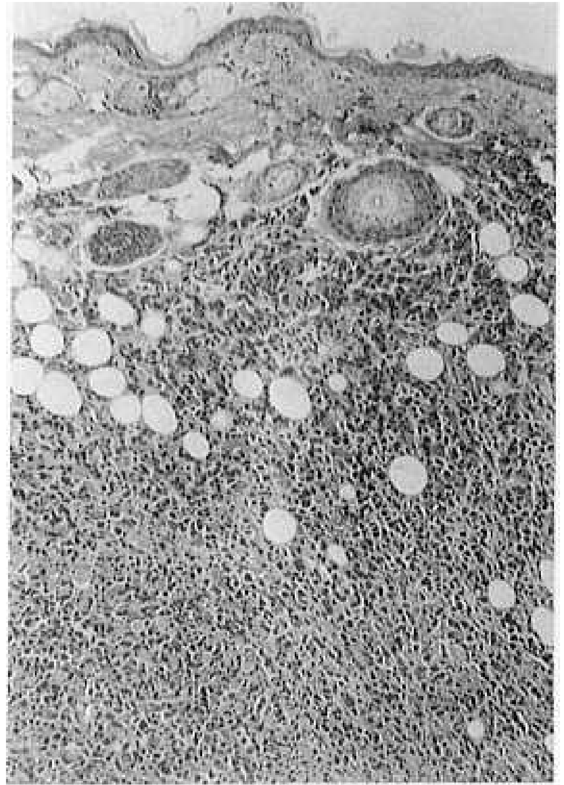

Fig. 4. Growing tumor mass after inoculation of S-180 cells into subcutaneous tissue (H&E×100).

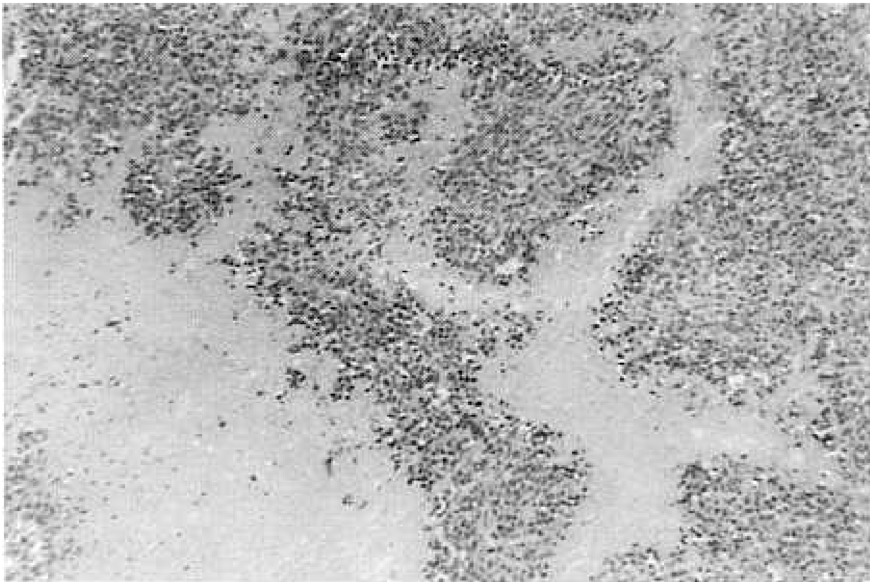

Fig. 5. Sarcoma showing geographic necrosis 2 days after PDT with CpD. (H&E, ×100).

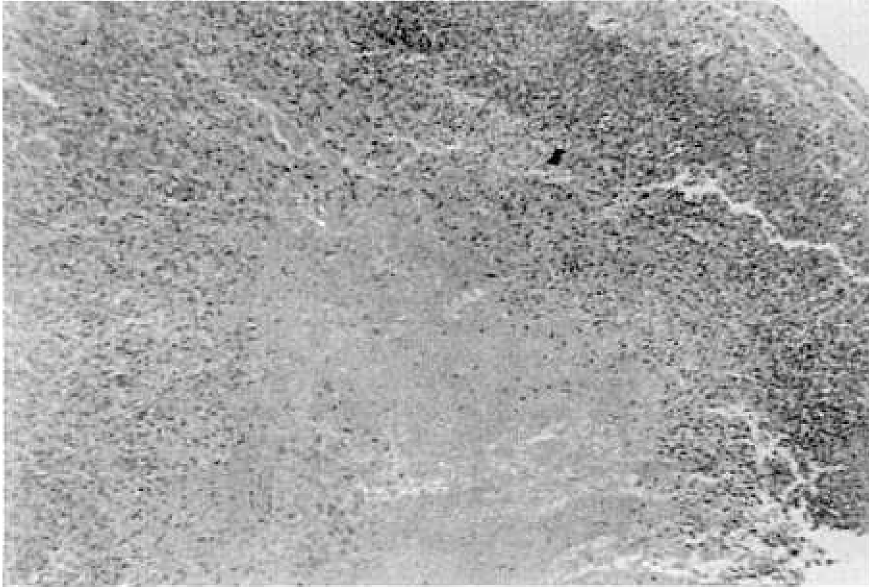

Fig. 6. Completely treated tumor showing central necrosis and surrounding inflammatory reactions 3 weeks after PDT with CpD, (H&E, ×100).

posttreatment revealed massive geographic necrosis in the tumor tissue. The findings at 3 weeks posttreatment revealed central necrosis and surrounding inflammatory reactions with no tumor cells. This series of events is depicted in Figures 4-6.

DISCUSSION

During the past 10 years, photodynamic therapy has been proved to be a promising new therapeutic modality in the treatment of cancer. Photodynamic therapy requires the combination of a photosensitizer and visible light to create the photodynamic effect since neither acting by itself is capable of creating cytotoxicity. In some cases, it may be a viable alternative to debilitating surgery, while in others, it may be the treatment of choice.

Ever since Dougherty first suggested the use of HpD, a light-sensitizing tumor localizing porphyrin, plus light to bring about selective tumor necrosis, the medical applications of this new therapeutic modality have been pursued vigorously (Dougherty *et al.* 1975, 1978).

Therefore, the most extensively studied photosensitizer for photodynamic therapy is HpD. This complex mixture of porphyrins is currently undergoing clinical trials to determine its efficacy in the treatment of neoplasms at selected sites. Preliminary studies have reported a response rate of up to 60% (Dahlman *et al.* 1983).

In spite of the demonstrated efficacy of HpD, there has been an intensive search for new photosensitizers (Nelson *et al.* 1987; Evensen and Moan 1987; Morgan *et al.* 1987). To be maximally effective, these should fulfill the criteria for the ideal photosensitizer which include: (1) should have no systemic toxicity, (2) should be taken up and retained only by malignant tissue, and (3) must absorb light and efficiently destory malignant tissue at wavelengths not absorbed by normal tissue (Morgan *et al.* 1987).

CpD is known to have moderate sized absorption bands with high cellular cytotoxicity at wavelengths of 670 nm (Lee *et al.* 1989), thus providing an advantage over the lower tissue penetrance of 630 nm used for HpD. Our study was undertaken to evaluate the photosensitizing potential of CpD with a significant absorption band located at 670 nm. This study describes the first successful "cures" resulting in long-term animal survival with CpD and light.

The results of our study suggest that CpD is an effective tumor photosensitizer in vivo. Our study showed that a 100% cure rate could be obtained at a 0.25mg intratumoral dose of CpD with 1h and 24h photodynamic therapy with red light. Moseng (1985) used hairless mice with subcutaneously transplanted Lewis lung carcinoma to study the effects of treatment

with HpD. His results showed high cure rates (70-90%) following a single treatment and showed that the high remission rate was obtained when using more than 0.1 mg of HpD. Similar results were obtained in our experiments using 0.25mg of CpD. Moseng (1985) used a 1000 W lamp and we used a 300 W lamp, but we were able to obtain similar results although it appears that the method of irradiating the light was different in these two cases. We irradiated the light 1-2 mm directly above the tumor area.

Initially the projector beam was used as the major light source; however, the dye laser has become a major light source nowadays (Dougherty *et al.* 1975; Wilson and Patterson 1986; McCaughan 1987). The advantage of using the dye laser is that it produces a specific wavelength applicable to the particular photosensitizer. Projecting the laser beam through a fiberopticscope makes it possible to have early diagnosis and treatment for the malignancies of the gastrointestinal tract (Yajiri *et al.* 1987), urinary tract (Benson *et al.* 1982), and tracheobronchial tree (Hayata and Kato 1983). If the dye laser had been used in these experiments, the same results might have been reached with a lesser dose of CpD.

While conducting this experiment, three separate experiments were conducted. One experiment was to find the LD 50 of CpD in ICR mice. Up to the dose of 150 mg of CpD, we were not able to find the LD 50 dose of CpD. Therefore, we concluded that CpD was quite a safe agent. In another experiment, we injected 0.125mg of CpD intratumorally, which was half the dose of CpD used in the original experiment.

The remission rate in this experiment was much lower than that of the original experiment. In the last experiment, PDT was conducted for 15 minutes 1h after the injection of HpD and 4/12 mice died in this experiment. While our study is promising, the studies on CpD are still in the early phase.

Therefore, we came to the conclusion that further study of PDT with intraperitoneal administration of CpD should be conducted considering factors such as treatment time interval, agents, dose of agent, dose of the light, light sources, etc.

Unanswered questions include delineation of light and drug dosimetry parameters, mechanisms of tumor localization, possible uptake in other organs such as liver, intestine, spleen, and kidney, as well as determination of PDT cytotoxicity.

It is hoped that future investigations will address these questions so that the role of CpD in the management of cancer can be fully defined.

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