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Original

A Basic Study of Intraoperative Photodynamic Therapy for Lung Cancer: Photodynamic Therapy for Lymphogenous Metastases in Nude Rats

Eiki MIZUTANI[1)]*, Hidenori INOUE[1)], Osamu SHIMADA[2)], Hirochika MATSUBARA[1)], Masami KOBAYASHI[3)] and Masahiko MATSUMOTO[1)]

[1)] *Second Department of Surgery, Yamanashi University of Medicine, Japan*

[2)] *Second Department of Anatomy, Yamanashi University of Medicine, Japan*

[3)] *Department of Technology, Tsukuba University, Japan*

Abstract: Background: We designed a new photodynamic therapy (PDT) protocol in which Pheophorbide *a* (Pba) accumulates in the lymph nodes following local administration around lung cancer tumors, followed by lobectomy and irradiation of the lymph nodes with lasers. As the first step, we evaluated whether administering PDT for metastatic lymph nodes is possible in a rat model.
Materials and Methods: Human lung squamous cell carcinoma (RERF-LC-AI) cells were subcutaneously injected into the foot pads of nude rats (F344/rnu-rnu) following X-irradiation. 3 mg/kg of Pba was injected around the tumors in the foot pads, and the Pba concentrations in the popliteal lymph nodes were measured chronologically from the absorption spectrum. The lymph nodes were irradiated with 670-nm light from a diode laser at 180 J/cm^2 for 600 seconds and examined histologically.
Results: The Pba concentrations in the lymph nodes reached a peak 40 minutes after local application and maintained approximately the same values until six hours. Widespread tumor cell necrosis was histologically evident in the specimens, and the relative proportion of the necrotic areas was 91.3 ± 8.1% (n=6).
Conclusions: Our findings suggest that Pba accumulates abundantly in metastatic lymph nodes following local administration into primary tumors and that administering PDT to treat the tumors is possible in a rat model.

Key Words: Non-Small-Cell Lung Carcinoma, Lymphatic metastasis, Pheophorbide *a*, photodynamic therapy

INTRODUCTION

Conventional photodynamic therapy (PDT) is primarily based on the differentiation of concentrations of photosensitizers between normal and tumor tissues using systemic administration of clinical therapy in patients with lung cancer[1-4)]. With this therapy, the use of large pharmaceutical doses is necessary in order to achieve flow throughout the entire body to reach adequate concentrations in the tumor. This results in the possibility for insufficient effects and strong adverse drug actions. Pheophorbide *a* (Pba) is one of the most favorable candidates for use as a photosensitizer in PDT because it can easily be prepared and purified from chlorophyll *a* and has a high extinction coefficient in the red re-

*1110 Shimokato, Chuo, Yamanashi 409-3808, Japan
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gion. Pba has a high affinity for lymph ducts, and its absorption coefficient is larger than that of other photosensitizers[5-12]. On the other hand, the lungs are characterized by the fact that pulmonary lymph ducts are concentrated in the pulmonary hilum, separate from the pulmonary veins.

Taking into consideration these two points, we designed a new PDT for use during surgery for lung cancer (Fig. 1). During surgery for lung cancer, the pulmonary veins are first separated so that the tumor cells are not scattered throughout the entire body (Fig. 1B). This allows the drug dosage in the body to be significantly reduced (Fig. 1C). In addition, the level of the drug in the body decreases, except for that in the lymph nodes, because the pulmonary lobe in which the drug is administered is removed. After lobectomy, a laser is used to irradiate the lymph nodes (Fig. 1D). As a first step, we evaluated how Pba accumulates in metastatic lymph nodes in a rat model and whether administering PDT for metastatic lymph nodes is possible.

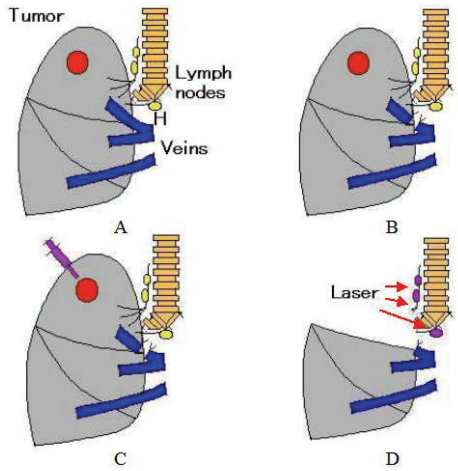

Fig. 1. New PDT during surgery for lung cancer. 1A: Schema of lung cancer in the right upper lobe. 1B: The pulmonary veins are first separated so that the tumor cells are not scattered throughout the entire body. 1C: The photosensitizer is injected around the tumor. The photosensitizer does not flow into the veins. 1D: The laser irradiates the lymph nodes after lobectomy. H: pulmonary hilum.

MATERIALS AND METHODS

Photosensitizer

Structurally, Pba (CAS 15564-29-6) is derived from chlorophyll a following the removal of phytol and magnesium by the actions of chlorophyllase or acid (Fig. 2). The crude pigments were purified by reversed-phase HPLC, and were finally converted to sodium salt to be soluble directly in water[13].

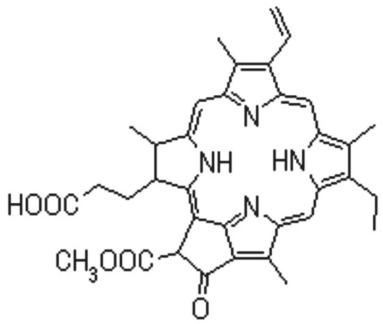

Fig. 2. The structural formula of Pba.

Tumor cells

RERF-LC-AI human lung squamous cell carcinoma cells were cultured in minimum essential medium containing 10% fetal bovine serum. The cells were grown as a monolayer at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. For implantation, the tumor cells were adjusted to a concentration of 1×10^6 viable cells per 0.5 m*l* of the same medium.

Animals and tumor model

Male nude rats (F344/rnu-rnu) were obtained at four weeks of age from Clea Japan and ac-

climated for one week before undergoing cell implantation. Five-week-old rats weighing 80-130 g were subjected to 500 rad of whole-body X-irradiation. Six hours later, the tumor cells were subcutaneously injected into the foot pads of the rats. The present model was newly established for this study, and the details have already been reported in the Japanese Journal of Lung Cancer[14]. The tumor cells were subcutaneously injected into the foot pads of the nude rats after X-irradiation. Two months later, metastasis to the popliteal lymph nodes occurred at a high rate. All animal experiments were approved by the University of Yamanashi Animal Care and Use Committee.

Measurement of the Pba concentrations in the popliteal lymph nodes

Under shading, 3 mg/kg of Pba was injected around the tumors in the foot pads two months after cell implantation. The dosage of Pba used was based on that reported by Fujishima[15]. The Pba concentrations in the popliteal lymph nodes were examined after 20 min, 40 min, 80 min, three hours and six hours. At these times, the lymph nodes were obtained, frozen promptly in liquid nitrogen and stored in a deep freezer at −80 °C. They were then placed in 1 m*l* of 99% methanol and crushed with a glass homogenizer. A further 4 m*l* of methanol was then added, and the samples were sonicated for 30 sec and left to stand for 2 hr at 0 °C to extract the Pba. After centrifugation at 4,000 rpm for 5 min, the supernatant was passed through a 0.22-um germ-free filter, and the absorption spectrum was measured with a spectrophotometer at an emission wavelength of 666 nm. The Pba concentration was determined from the absorption spectrum using the Lambert-Beer formula.

Photodynamic therapy

Under shading, 40 min after injection of Pba, the 4 popliteal lymph nodes were irradiated with 670-nm light from a diode laser at 180 J/cm^2 (300 mW/cm^2) for 600 sec. Next, 80 min after injection, the 6 lymph nodes were irradiated under the same conditions. In addition, irradiation with the laser beam was confirmed by observing fluorescence of Pba in the lymph nodes without wavelength of nearby 670 nm. After laser irradiation, the rats were again kept under shading. Forty-eight hours after irradiation, the lymph nodes were excised, fixed in 10% formalin, stained with H&E and examined histologically to study the effects of PDT. We examined the lymph nodes across their largest cut surface and determined the relative area of necrosis (= area of tumor cell necrosis / (necrosis + area of non-necrotic tumor cells)) using an optical microscope.

Statistical analysis

The significance of differences was evaluated using the Mann-Whitney U-test, with *P* values lower than 0.05 being considered significant.

Results

Tumor growth and metastasis to popliteal lymph nodes

The take-rate of the tumors in the foot pads was high, as confirmed on histopathological examination of H&E-stained samples showing that the characteristics of the tumors did not change. The tumors grew gradually after a latent period of approximately one week. Two months after cell implantation, the largest of the popliteal lymph nodes measured 6 mm in diameter and the smallest lymph node measured 3 mm in diameter. Samples of the popliteal lymph nodes were easily obtainable by naked eye observation. Metastasis to the popliteal lymph nodes

occurred at a high rate, and a histopathological study showed invasion of the tumor cells into the cortex and medulla occupying over one half of the node (Fig. 3).

Pba concentrations in the popliteal lymph nodes

The popliteal lymph nodes were observed to be dyed 5 min after local injection of Na-Pba around the tumors, and accumulation of Pba was confirmed with the naked eye (Fig. 4). The absorption spectrum of the popliteal lymph nodes after local injection of Pba exhibited the same two major peaks as that of Pba. The retention-time course curve for the lymph nodes is shown in Fig. 5. The concentrations of Pba in the popliteal nodes became maximal within 40 min of local application, reaching 209 ± 128 pmol/mg (n=6), and maintained approximately the same values until six hours.

Histological appearance of the lymph node metastases after PDT

Widespread tumor cell necrosis was histologically evident in the specimens sampled 48 hr after PDT (Fig. 6). Some specimens showed complete cell necrosis with a total loss of tumor cells. The relative proportion of the necrotic areas resulting from PDT 40 min after local injection of Pba was 56.7 ± 19.9% (n=4). Because allowing time for the drug to be incorporated into the cells might be necessary, we examined the specimens 80 min after local injection. The relative proportion of the necrotic areas resulting from PDT 80 min after local injection was 91.3 ± 8.1% (n=6) (Fig. 7). The most marked damage in the tumor cells caused by PDT was evident at 80 min.

DISCUSSION

The ideal photosensitizer for use in PDT is one that accumulates specifically in tumor cells and exhibits high cytotoxicity. Pba has a Soret band of approximately 400 nm and a Q absorption band in the visible light range. The Q absorption band is approximately 667 nm, which is longer than that of Photofrin. Furthermore, the absorbance coefficient of Pba is very large. A

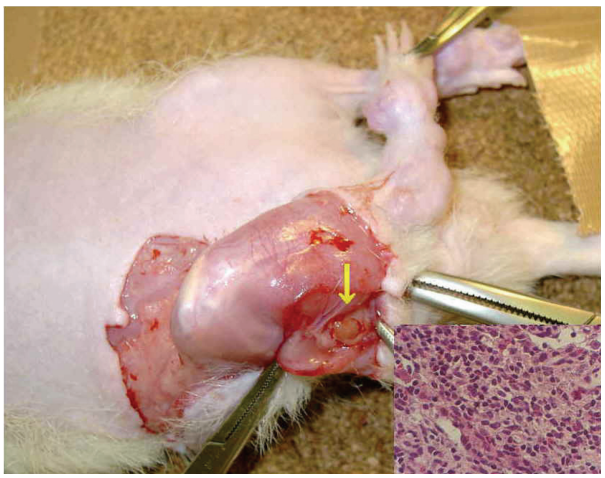

Fig. 3. Macrograph of metastatic popliteal lymph nodes. A light micrograph (H&E, × 200) showing lymph node metastases.

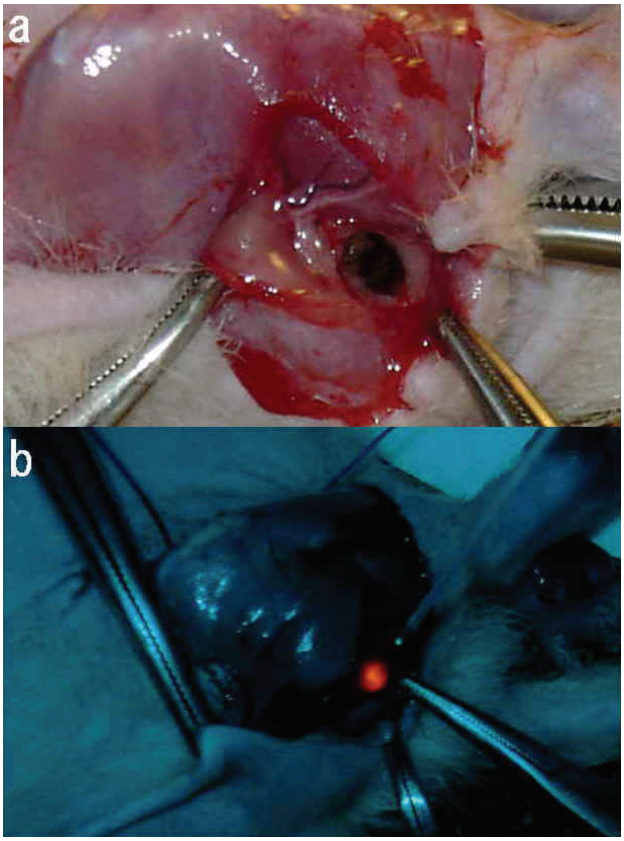

Fig. 4. a: Dyeing of the popliteal lymph nodes (five minutes after local injection of Pba). The popliteal lymph nodes were dyed five minutes after local injection of Pba around the tumors, and accumulation of Pba was confirmed with the naked eye. b: Fluorescence findings of the popliteal lymph nodes using laser irradiation (shading light of 670-nm wavelength). Fluorescence of Pba was observed in the lymph nodes.

long-wavelength laser can penetrate tissue easily, thus allowing for PDT in deeply seated tumor cells. A high concentration of Pba has been reported by our group to accumulate in human lung squamous cell carcinoma RERF-LC-AI cells after local injection of Pba, which allows sufficiently effective use of PDT *in vitro*[16-18]. On the other hand, Pba is known to be very fat-soluble, showing marked affinity for lymph ducts. Therefore, we considered that Pba would accumulate easily in the lymph node metastases after local injection of Pba around the tumors. We herein reported the histological effectiveness of PDT for treatment of lymph node metastases in a nude rat model.

It has been reported that the maximum concentration of Pba in tumor cells is 10.2 ± 2.8 pmol/mg after intravenous injection at a dose of 20 mg/kg[10]. In comparison, the Pba concentrations in the popliteal lymph node metastases

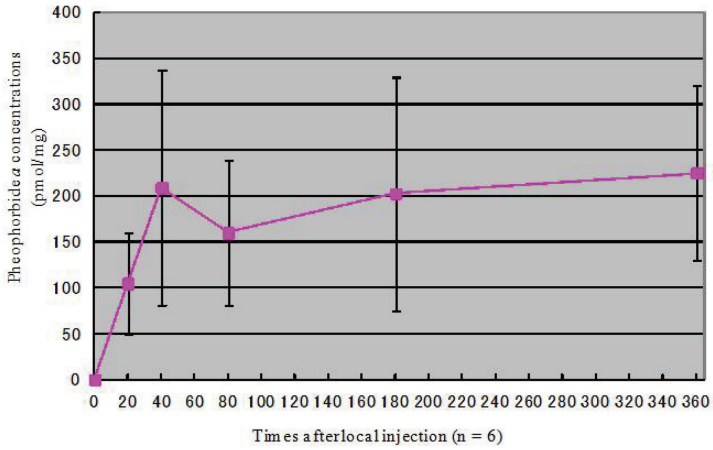

Fig. 5. The Pba concentrations in the popliteal lymph nodes after local injection of Pba. Each point indicates the mean ± SEM of five to seven animals. The concentrations of Pba in the popliteal lymph nodes reached a peak 40 min after local application and maintained approximately the same levels until six hours.

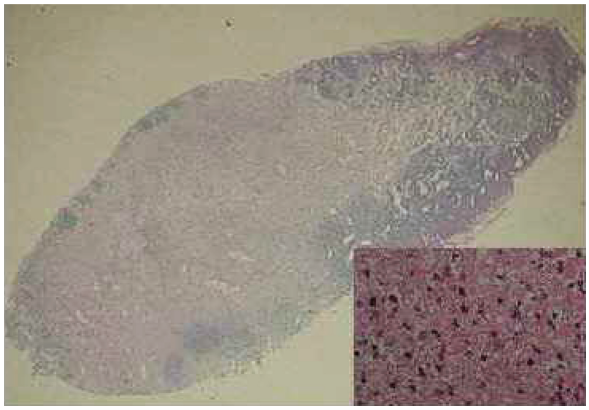

Fig. 6. Light microscopic findings of the popliteal lymph nodes following PDT (H&E, × 10). Histological findings of the popliteal lymph nodes following PDT (H.E., × 200). The tumors were sampled 48 hr after a 180 J/cm^2 PDT session with a 670 nm laser with 3 mg/kg of Pba. There was evidence of complete cell necrosis with an entire loss of the usual arrangement of the tumor cells.

after local injection were over 100 times greater. In the present model, Pba showed marked accumulation in the target lymph nodes. In terms of damage to tumor tissue, it has been reported that the local curative effects of PDT are stronger than those of chemotherapy. In addition, PDT with Photofrin for brain glioma reportedly produces complete necrosis of 11.8 ± 6.5% and focal necrosis of 59.4 ± 3.7% of the tumor cell area[19]. The corresponding rate of complete

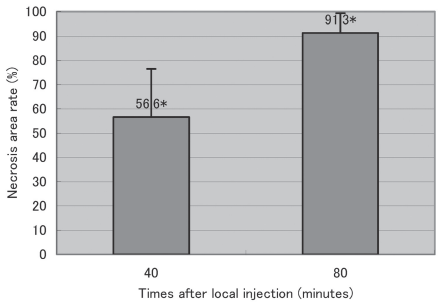

Fig. 7. The necrosis area rates following PDT at 40 min (n=4) and 80 min (n=6) after local injection of Pba. PDT with Na-Pba induced strong damage in the tumor cells, especially at 80 min. *P<0.05

necrosis in the present experimental trial of PDT was 91.3±8.1%, with little focal necrosis. Although the 2 experiments are not directly comparable because of differences in the target cells used, PDT with Pba induced stronger damage in the tumor tissue. Therefore, Pba appears to show promise as a new photosensitizer since it has few side effects and a high cytotoxity.

For patients with early lung cancer without distant metastasis, direct cancer extension to other organs or mediastinal lymph node metastasis, surgery is the first-choice treatment. The current normal surgical method is lobectomy and systematic hilar and mediastinal lymph node dissection. If a photosensitizer could be initially injected around the primary lung tumor followed by PDT after lobectomy and systematic lymph node dissection (or after lobectomy alone), then administering adjuvant therapy for metastases in lymph nodes and lymph ducts, which cannot be resected completely with surgery, would become possible. As for the effects of adjuvant PDT, it has been reported that rats subjected to PDT after resection of implanted breast cancer tumors show better outcomes than rats subjected to resection alone[20]. Therefore, it is expected that the use of PDT with Pba along with surgery will improve the long-term prognoses of patients with lung cancer.

In conclusion, Pba accumulates abundantly in metastatic lymph nodes following local administration around primary tumors, and administering PDT to treat these tumors is thus considered to be possible and effective in a rat model.

Acknowledgement

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