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Surface Layer-Preserving Photodynamic Therapy (SPPDT) in a Subcutaneous Mouse Model of Lung Cancer

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Abstract

Background and Objectives—Photodynamic therapy (PDT) may be a less invasive treatment for lung cancer. Our newly developed surface layer-preserving PDT (SPPDT) technique enables us to irradiate deep tumor while preserving the overlying tissue. The aim of this basic study was to verify that the SPPDT technique might be applied to lung cancer.

Study Design/Materials and Methods—PDT with talaporfin sodium was performed using a pulsed laser with different pulse dose rates (PDRs, 2.5–20.0 mJ/cm²/ pulse) in a mouse model of subcutaneous tumor. To mimic the tracheal wall structure and a thoracic tumor in the tracheobronchus, we also made a mouse model in which a piece of swine cartilage was placed between the dermis and the tumor, and PDT was carried out 2 weeks after implantation. In both experiments, the tissue samples were collected 48 hours after PDT and evaluated microscopically.

Results—SPPDT using a high-PDR laser damaged the underlying tissue but left the superficial tissue intact in the mouse subcutaneous tumor model. In SPPDT, a higher PDR produced a thicker layer of intact superficial tissue than a lower PDR, while a lower PDR produced a deeper layer of damaged tissue than a higher PDR. SPPDT was also able to preserve the superficial tissue and to damage the tumor tissue beneath the cartilage implant.

Conclusion—SPPDT was able to damage tumor beneath the superficial normal tissue layer, which included tracheal cartilage in the mouse model. The thickness control of SPPDT was provided by controlling laser pulse intensity. SPPDT is a new technology, whose future potential is unknown. The initial clinical application of this technology could be endoscopic treatment (e.g., palliative therapy of thoracic malignancies *via* bronchoscopy).

Keywords

photodynamic therapy; taloporphin sodium; pulsed laser; surface layer preservation; tracheobronchial cancer; lung cancer; Lewis lung carcinoma; pig tracheal cartilage; mediastinal lymph node

INTRODUCTION

More than 1 million people worldwide die of lung cancer every year [1]. The three main pillars of treatment for lung cancer are surgery [2,3], chemotherapy, and radiotherapy; however, only 30% of all patients (mainly those with early-stage lung cancer) are candidates for surgery. Most patients are diagnosed as already advanced cases and have metastases to lymph nodes and/or distant organs [4]. Mediastinal lymph node swelling as a result of metastasis sometimes causes tracheal stenosis [5,6], recurrent nerve paralysis [7,8], or superior vena cava syndrome [9,10]. Chemoradiotherapy is often indicated for lung cancer with mediastinal lymph node metastasis [11–14]. However it can sometimes cause the serious side effects of radiation pneumonitis, esophagitis, and pancytopenia [15].

Photodynamic therapy (PDT) is a newly developed less-invasive treatment for lung cancer [16–18]. Though generally reserved for use in patients with locally advanced tumors requiring palliation *via* debulking of endobronchial tumor, PDT has been used in patients with early stage cancers who are not candidates for curative resection [19–21], or preoperatively in an effort to downstage lung tumors in advance of resection [19,20]. It is common to use a CW laser as a light source for PDT because of its wide availability. On the other hand, PDT with a pulsed light source can affect deeper tissue layers than PDT using a continuous wave laser [22,23]. Okunaka et al. [22] performed an *in vivo* PDT experiment with hematoporphyrin derivative on a mouse kidney sarcoma implanted in mice and compared pulsed and CW irradiation. A pulsed excimer laser pumped dye laser irradiation system was shown to give three times more depth of necrosis than an equivalent CW Ar laser pumped dye laser irradiation system. Arai et al. [24] reported they serendipitously had discovered a new pattern of tissue preservation while exploring patterns of cytotoxicity by a high peak power pulsed laser, which was presented as preliminary work at IPA Eighth World Congress in 2001. They noticed a layer of normal tissue was preserved above the PDT damaged tissue in an animal model of cancer. They also suggested that the surface layer-preserving PDT (termed SPPDT) effect could not be explained by assuming that the pulse dose rate (PDR) and total light dose affected the singlet oxygen production, the oxygen consumption or the overall photocytotoxicity [25]. Therefore they hypothesized that changes in the circulation within the tissues during PDT was the cause of the phenomenon as this has been reported to be a critical feature of PDT [26–28]. A trans-tracheobronchial approach would allow the easiest approach to irradiate metastatic lesions in the mediastinal lymph nodes; therefore, SPPDT might be applicable to bronchoscopic laser PDT of mediastinal metastatic lesions if the prevention of damage to the tracheobronchial wall could be achieved. In the present report, we experimentally examined the feasibility of SPPDT using two mouse subcutaneous tumor models.

MATERIALS AND METHODS

Cells

Lewis lung carcinoma (LLC) cells (ATCC, Manassas, VA), which originated spontaneously from a mouse lung, were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 1% penicillin, and 1% streptomycin (all from Invitrogen, Life Technologies Corp., Carlsbad, CA). These cells were maintained at 37°C in an atmosphere of 5% carbon dioxide/95% air.

Animals

Six-week-old homozygous BALB/c male nude mice weighing 20–25 g were obtained from Clea Japan, Inc. (Tokyo, Japan). The animals were maintained under laminar airflow conditions in the Laboratory for Animal Experiments, School of Medicine, Keio University. The care and handling of the animals were done in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Keio University. Although LLC cells are syngenic to C57BL/6 mice, immunosuppressed nude mice were chosen for the present studies since the swine cartilage implanted could have caused an immune response.

Experimental Model

Mouse subcutaneous tumor model—Nude mice were anesthetized through an intramuscular injection of a cocktail containing ketamine (60 mg/kg) and xylazine (6 mg/kg) in sterile normal saline prior to surgery and tumor implantation. The LLC tumor cells (1×10^7) were suspended in 20 μ l of phosphate buffered saline containing 10 μ l of Matrigel®, a basement membrane matrix, and injected into the subcutaneous tissue above the left hindleg. After injection, the subcutaneous tumor was allowed to grow to a size of about 10 mm diameter prior to animal sacrifice and tumor resection. The resected tumor was then minced into 1-mm² cubes. After the skin above the left hindleg of a secondary host mouse was incised 1.5 mm, autologous tumor from the first host was transplanted subcutaneously to a portion about 7 mm away from the incision of the secondary host. Two weeks after implantation, the tumor had grown to a size of about 10 mm diameter and was subsequently used for the experimental study.

Mouse subcutaneous tumor with cartilage model—The main aim of this research was to verify the possibility of SPPDT application to the trans-tracheobronchial treatment of mediastinal lesions. To mimic the trachea structure, that is, the epithelium, subepithelium, tracheal cartilage, and mediastinal lymph node, we used a mouse model in which a piece of cartilage was placed between the dermis and the tumor.

After removing the peripheral tissue from the tracheal cartilage of a swine (obtained from Tokyo Shibaura Zouki Co., Ltd., Tokyo, Japan) and washing with PBS, it was cut and shaped into 7 × 10-mm blocks (average thickness: 0.8 mm). After the skin of the left mouse hindleg was incised about 8 mm, the cartilage block was then simultaneously implanted subcutaneously above a 1-mm square tumor cube, in the same manner as described above. Two weeks after the implantation in the secondary host, the tumor had grown to a size of about 10 mm diameter and was subsequently used for the experiments.

Photosensitizer

Talaporfin sodium was provided by Meiji Seika Kaisha, Ltd. (Tokyo, Japan). The absorbance peak of this photosensitizer in the red region was measured to be 664 nm using a solvent composed of a mixture of RPMI1640 medium (without phenol red) supplemented with 10% fetal bovine serum, 20 IU/ml of penicillin, and 20 μ IU/ml of streptomycin (all from Invitrogen).

Laser Light Source

To excite the talaporfin sodium, an excimer-pumped dye (EPD) laser (EDL-1; Hamamatsu Photonics Co., Ltd., Hamamatsu, Japan) and a single silica fiber of 600 μ m (Hamamatsu Photonics Co., Ltd.) were used as the pulsed light source. A laser dye consisting of 0.5-mM Rhodamine 640 and 0.05-mM Oxazine 740 dissolved in ethanol was used. The wavelength, repetition rate, and pulse duration of the laser were fixed at 664 nm, 40 Hz and 9 nanoseconds, respectively.

Talaporfin Sodium Distribution Experiment

Under anesthesia, five mice were intravenously injected in the tail vein with talaporfin sodium (5.0 mg/kg body weight). The mice were shielded from light to avoid room light-induced photosensitivity. The mice were sacrificed 1 hour after injection with talaporfin sodium and the tumors were then removed, sliced into 50- μ m-thick sections, and examined using fluorescence microscopy. The excitation wavelength was 410 nm, while the emission wavelength range was over 550 nm.

Performing PDT

PDT in the mouse subcutaneous tumor model—The mice were treated in the same manner as described above and 1 hour after talaporfin sodium administration, an area of the mouse skin (5 mm in diameter) over the implanted tumor was irradiated using the EPD laser after the mouse had been anesthetized. The PDRs were 2.5, 5.0, 10.0, and 20.0 mJ/cm²/pulse (for each PDR group n = 7). The total light dose was 50 J/cm². At 3 minutes before PDT, just after PDT, and 48 hours after irradiation, the skin blood flow volume in the irradiated field was measured using a laser Doppler blood perfusion monitor (PeriFlux, PERIMED, Sweden; Jarfalla, Sweden).

PDT in the mouse subcutaneous tumor with cartilage model—Irradiation was performed in the same manner as described above for the mouse subcutaneous tumor model. We used the EPD laser (PDR, 10.0 mJ/cm²/pulse; total light dose, 50 J/cm²; n = 6).

Evaluation of PDT Effect

The PDT-treated tissue sample was then removed from mice sacrificed 48 hours after irradiation, sliced into 3- μ m-thick sections, and stained with hematoxylin and eosin (H&E). The effect of PDT was observed using a magnified microscopic image. The preserved layer thickness and the damaged layer thickness were defined as the average thickness of the preserved layer and the damaged layer on five vertical lines drawn from the center of the skin irradiation field and its bilateral points at distances of 0.125 and 0.250 mm from the center point on the sections. In the PDT group with the mouse subcutaneous tumor model, the thickness of the both damaged and preserved layers was measured.

Statistical Analysis

Data are expressed as mean $\pm$ SD and were analyzed by *t*-test. The values of *P* < 0.05 were considered statistically significant.

RESULTS

Talaporfin Sodium Distribution

Figure 1A shows the H&E-stained control images and Figure 1B shows the corresponding fluorescence microscopic images. There was a homogeneous distribution of talaporfin sodium in the tumor of moderate fluorescence intensity, whereas a higher accumulation was observed in the fascia as an intense fluorescence (Fig. 1B).

PDT Effect in Mouse Subcutaneous Tumor Model With Pulsed Irradiation

PDT with a PDR of 2.5 mJ/cm²/pulse produced a uniformly damaged set of tissue layers extending from the skin surface to the deepest margin of the tumor (Fig. 1C). However PDT with a PDR of 10.0 mJ/cm²/pulse produced a different pattern of damage, with a damaged layer visible beneath the preserved layer in the mouse subcutaneous tumor (Fig. 1D). In the preserved normal tissue upper layer, no cell morphological changes were observed. In the deeper damaged tumor layer, however, cells with nuclear pyknosis and karyorrhexis and a

degenerative cellular outline or minimal preservation of the basic cellular shape were observed.

A preserved layer appeared when a PDR of 10.0 or 20.0 mJ/cm²/pulse was used, but not when a PDR of 2.5 or 5.0 mJ/cm²/pulse was used. A higher PDR produced a wider preserved layer thickness than a lower PDR ($P < 0.05$; Fig. 2A).

The PDT damage reached the deepest margin of the tumor when a PDR of 2.5 or 5.0 mJ/cm²/pulse was used; in this situation, the total damaged layer thickness was equivalent to the thickness from the skin surface to the deepest margin of the tumor. A low PDR produced a wider damaged layer thickness than a high PDR ($P < 0.05$; Fig. 2B).

When a preserved layer was achieved with a high PDR, the average skin blood flow volume of the skin in the irradiated field decreased to half of the baseline value immediately after PDT but had returned to the baseline value at 48 hours after PDT (Fig. 3). In cases without a preserved layer, the average skin blood flow volume remained relatively stable immediately after PDT but had decreased at 48 hours after PDT (Fig. 3). Blood flow volume measured just after PDT was significantly ($P < 0.05$) higher when there was no preserved layer (2.5 mJ/cm²) than when there was a preserved layer (10 mJ/cm²), however, when measured 48 hours after PDT, the situation was reversed and blood flow volume was significantly ($P < 0.05$) higher in the preserved layer case.

PDT Effect in the Mouse Subcutaneous Tumor Model With Cartilage

PDT with a PDR of 10.0 mJ/cm²/pulse resulted in the preservation of the skin and caused damage to the tumor tissue beneath the cartilage (Fig. 4). Significant amounts of tumor damage were achieved underneath the implanted cartilage layer, although the depth of tumor damage may have been somewhat smaller than that seen without the implanted cartilage layer under the skin.

DISCUSSION

We were able to obtain preservation of an intact surface normal tissue layer above the necrotic tumor layer in this mouse subcutaneous tumor model. This preserved layer has not been reported in the case of CW irradiation in Talaporfin sodium mediated PDT [29–33] or in PDT mediated by other photosensitizers [19,34–36]. Talaporfin sodium is a second-generation hydrophilic chlorin-based photosensitizer that has been reported in basic and clinical studies to mediate the treatment of various malignant tumors and age-related macular degeneration [37–40]. The Q band absorption peak of Talaporfin sodium is located at the longer visible red wavelength (664 ± 10 nm), providing the advantage of large penetration depth compared to shorter wavelengths such as green or yellow light. In our PDT experiment, laser irradiation with a low PDR (2.5 or 5.0 mJ/cm²/pulse) did not produce a preserved layer, while irradiation with a high PDR (10.0 or 20.0 mJ/cm²/pulse) produced a preserved layer in the skin and the superficial part of the tumor regardless of the homogeneity of the talaporfin sodium distribution. Thus, a high-pulsed laser light intensity may be required for SPPDT. In the group with a preserved layer produced by high PDR, the blood flow reduction just after PDT and the late recovery of irradiated tissue was observed while it was not seen in a group without the preserved layer. What is the mechanism of this SPPDT effect? Some reports have mentioned a blood flow volume reduction and the late recovery of irradiated tissue after pulsed laser irradiation [41,42]. They predicted that photocoagulation in micro vessels appeared during PDT irradiation, and hypothesized that restoration of vascular function by the reperfusion of existing incompletely photocoagulated microvasculature occurred. In our study, we also observed changes in blood flow kinetics in the skin of three of seven mice (four mice were excluded due to body movement interfering

with the precise measurements) in PDT with a high PDR (10.0 or 20.0 mJ/cm²/pulse). We believe the cause of the phenomenon is due to the microvessel photo-coagulation and the reperfusion induced by pulsed laser irradiation. The fact that laser irradiation with a low PDR (2.5 or 5.0 mJ/cm²/pulse) did not produce the blood flow volume reduction showed a high pulsed laser light intensity is required for blood flow reduction. In other words, there is a threshold where blood flow reduction occurs between 5.0 and 10.0 mJ/cm²/pulse. Channal et al. [42] reported blood volume reduction was not seen in the case of PDT using CW irradiation (total light dose 50 J/cm²). These observations are in agreement with the lack of reports that a preserved layer was observed in PDT using CW laser [19,29–36]. The blood flow volume reduction produced by high-pulsed laser intensity caused a reduction of talaporfin sodium concentration, and of oxygen and lower PDT effect in the skin and upper layer of the tumor. This mechanism therefore could have produced a preserved layer. The reason the preserved layer appears in skin in spite of higher accumulation of talaporfin sodium in skin than tumor can be explained by this mechanism. The reason blood flow volume reduction was observed 48 hours after PDT with a low PDR correlates with skin necrosis produced by CW PDT. As the laser light intensity increases, the photons reach a deeper layer of the irradiated tissue. This is a reason the preserved layer thickness is deeper in laser irradiation with a PDR of 20.0 mJ/cm²/pulse than 10.0 mJ/cm²/pulse. In our *in vitro* study, we measured cell viability between two conditions (PDR 2.5 mJ/cm²/pulse, irradiation time 200 seconds; 10.0 mJ/cm²/pulse, irradiation time 50 seconds) where the total light doses (20 J/cm²) were same. In spite of same total light dose, the condition, which had longer irradiation time produced lower viability (data not shown). From this fact, we predict the reason the damaged layer is deeper in PDT with PDR of 10.0 than 20.0 mJ/cm²/pulse is due to longer irradiation time in PDT with PDR of 10.0 mJ/cm²/pulse than 20.0 mJ/cm²/pulse.

In our experiments, the skin temperature in the irradiation field during PDT with PDR of 2.5 and 10.0 mJ/cm²/pulse increased by an average of 1.3 and 2.2°C, respectively. The highest temperatures reached were 23.3 and 27.5°C, respectively. Therefore we think thermocoagulation [43,44] was not involved in the appearance of the preserved layer.

The tissue hypoperfusion caused by high PDR laser irradiation and irradiation time might give rise to the changes in the preserved layer thickness and the damaged layer thickness, suggesting that the regulation of the preserved layer thickness and the damaged layer thickness might be feasible by controlling the laser intensity during SPPDT.

To mimic the trachea structure, that is, the epithelium, subepithelium, tracheal cartilage, and mediastinal lymph node, we used a mouse model in which a piece of cartilage was inserted between the dermis and the tumor. In this mouse subcutaneous tumor model, a PDR of 10 mJ/cm²/pulse was chosen because it preserved about 0.9 mm deep of the tissue as the average, which is almost the same as the average distance between the cutaneous surface and the surface of implanted pig cartilage. PDT with a PDR of 10.0 mJ/cm²/pulse did not affect the skin and the subcutaneous tissue but successfully damaged the tumor tissue beneath these structures. If CW laser or pulsed laser with a low PDR (2.5 or 5.0 mJ/cm²/pulse) were used, the skin in irradiation field would be expected to become necrotic.

According to measurements made in nine Japanese cadavers (mean age of 83 years at the time of death) at our hospital, the average thicknesses of the human trachea, left main bronchus and right main bronchus were 1.55 mm (range, 1.7–1.3 mm), 1.37 mm (range, 1.6–1.0 mm), and 1.31 mm (range, 1.4–1.1 mm), respectively, and fairly swollen mediastinal metastatic lesions attach to the tracheobronchial cartilage through an intermediary connective tissue sheath (with an average thickness of 0.15 mm; range, 0.1–0.2 mm). The total thickness of the epithelium and subepithelium together is 0.1 mm. Human

cartilage is somewhat thicker than swine cartilage; however, the actual damage to the cartilage of the tracheobronchial wall during SPPDT is expected to be minimal because of the high pulsed laser light intensity and the poor accumulation of talaporfin sodium as a result of the limited blood flow in the cartilage. Therefore in possible clinical application, preservation of overlying cartilage may be achieved by three interlocking effects (i) low PS accumulation in cartilage; (ii) low blood flow in cartilage restricting oxygen availability; and (iii) the SPPDT effect at high pulse energies.

In this model, the skin and the cartilage were used to mimic the tracheal wall, while the tumor tissue was used to mimic mediastinal lymph node metastasis or other peritracheal tumors. These results suggest that bronchoscopic SPPDT might produce tumor volume reduction in a mediastinal lymph node metastasis or other peritracheal tumors beyond the trachea wall without damaging the trachea itself. Several paramalignant clinical conditions, such as superior vena cava syndrome, tracheal stenosis, esophageal stenosis, are caused by mediastinal lymph node swelling or mediastinal tumor, therefore, this technique would be applicable for palliative therapy of thoracic malignancies, and also other malignant tumors in the proximity of accessible tubular organs. However considerable further study is needed to establish whether it will indeed be clinically useful.

In further studies, in order to explore the production PDT damage in deeper layers and verify this phenomenon is applicable to a new endoscopic SPPDT modality, we will perform SPPDT on mediastinal lymph node through the trachea and estimate a response rate, overall survival rate, and adverse effect using larger animals than mice, and studying various PDRs and total light doses.

CONCLUSION

SPPDT could allow underlying tumor to be damaged by PDT while allowing the overlying normal tissue to be preserved. It is thought that the SPPDT mechanism involves vascular occlusion by thrombosis. Further study to refine the spatial control by varying laser parameters is needed for its clinical application.

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Fig. 1.

A: Hematoxylin–eosin (H&E)-stained control image of an implanted subcutaneous tumor. **B:** Fluorescence microscopic image of the tumor 1 hour after the injection of 5 mg/kg of talaporfin sodium. The excitation wavelength was 410 nm. The measured wavelength was over 550 nm. The bright portion in the fluorescence image represents the accumulation of talaporfin sodium. The homogeneous distribution of talaporfin sodium was confirmed in the tumor as moderate intensity. A higher talaporfin sodium accumulation was observed in the fascia as an intense fluorescence, compared to a fainter signal within the tumor. Auto-fluorescence appeared in the hair follicles and faintly in the fascia on fluorescence microscopic images without talaporfin sodium injection as a control (data not shown). **C:** H&E-stained image of a typical subcutaneous tumor irradiated using a pulsed laser (pulse dose rate [PDR], 2.5 mJ/cm²/pulse; total light dose, 50 J/cm²) at 1 hour after the injection of talaporfin sodium (5.0 mg/kg, intravenously). The tumor was excised 48 hours after the laser irradiation. Cells with nuclear pyknosis and karyorrhexis and a degenerative cellular outline or minimal preservation of the basic cellular shape were observed from the skin surface to the deepest margin of the tumor (damaged layer) on the image of the tumor. **D:** H&E-stained image of a typical subcutaneous tumor irradiated using a pulsed laser (pulse dose rate

[PDR], 10.0 mJ/cm²/pulse; total light dose, 50 J/cm²) at the same manner as described above. The degeneration or necrosis of the cell walls or nuclei caused by the photodynamic therapy (PDT) was observed in the layer indicated between the two lines of black arrowheads (damaged layer) on the image of the tumor, while no effect was observed in the superficial (preserved layer). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/lsm>]

Fig. 2.

A: Changes in the preserved layer thickness with variable PDR irradiation (2.5, 5.0, 10, or 20 mJ/cm²/pulse). When the PDR was 10.0 mJ/cm²/pulse or more, the preserved layer thickness was deepened as the PDR increased. **B:** Changes in the damaged layer depth with variable PDR irradiation (2.5, 5.0, 10, or 20 mJ/cm²/pulse). The PDT effect (PDRs, 2.5 and 5.0 mJ/cm²/pulse) reached to the deepest margin of the tumor. The damaged layer thickness narrowed as the PDR increased. PDT was performed using a pulsed laser (total light dose, 50 J/cm²) 1 hour after the injection of 5.0 mg/kg of talaporfin sodium. The tumor was excised 48 hours after the laser irradiation.

Fig. 3.

Changes in the skin blood flow volume in the pulsed laser-irradiated field of the PDT model. The laser Doppler perfusion monitor measured the skin's blood flow volume before PDT, just after PDT, and at 48 hours after PDT (*X*-axis). The *Y*-axis represents the percent change in the skin's blood flow volume relative to the value for the pretreatment state. In cases of PDT forming preserved layer the skin blood flow volume decreased just after PDT. Flow was restored at 48 hours after PDT. In contrast, the skin blood flow volume in cases of PDT not forming a preserved layer increased slightly just after the PDT and had decreased at 48 hours after PDT. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/lsm>]

Fig. 4.

H&E-stained image of a typical subcutaneous tumor with a cartilage model specimen irradiated using a pulsed laser (PDR, 10 mJ/cm²/pulse; total light dose, 50 J/cm²) at 1 hour after the injection of 5.0 mg/kg of talaporfin sodium. The tumor was extracted 48 hours after the laser irradiation. Tissue damage caused by the PDT was not observed in the skin, the subcutaneous tissue, or the cartilage, while the tumor tissue beneath the cartilage was damaged. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/lsm>]