Photodynamic Therapy Using Pheophorbide a and Nd:YAG Laser

Ichiro FUJISHIMA, Tsuneo SAKAI, Tokutaro TANAKA, Hiroshi RYU*, Kenichi UEMURA*, Yuriko FUJISHIMA**, Kentaro HORIUCHI***, Norio DAIKUZONO**** and Yoshio SEKIGUCHI****

Department of Neurosurgery, Seirei Mikatabara General Hospital, Hamamatsu, Shizuoka; Departments of *Neurosurgery, **Microbiology and Immunology, and ***Chemistry, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka; ****Surgical Laser Technologies Japan Co., Ltd., Tokyo

Abstract

The authors describe a new photodynamic therapy (PDT) method for malignant brain tumors. Pheophorbide a (Ph-a), the photosensitizer, has low toxicity, causes no skin sensitization and is activated with an acoustic Q switched neodymium yttrium-argon-garnet (Nd:YAG) laser which achieves deep tissue penetration. The Ph-a distribution in Fisher 344 (F344) rats bearing rat T9 glioma at 24 hours after intravenous injection was very low in the normal brain tissue, but significantly higher in the T9 glioma giving a tumor to normal brain tissue concentration ratio of 7.5:1. The in vitro survival rate of T9 glioma cells pretreated with Ph-a was 68.8 ± 5.4% after laser irradiation for 20 minutes, significantly lower than in the control groups. This indicates that Ph-a was activated with the acoustic Q switched Nd:YAG laser causing the photodynamic effect. The survival rate after Ph-a pretreatment and laser irradiation in a waterbath at 44.0°C was further reduced to 15.8 ± 3.3%. In vivo PDT studies using T9 glioma cells inoculated into the dorsal region of F344 rats showed tumor eradication in four of six rats. The combination of PDT and laser hyperthermia produced tumor eradication in all six rats. The combination of PDT and hyperthermia is a promising method for tumor treatment.

Key words: photodynamic therapy, Nd:YAG laser, pheophorbide a, laser hyperthermia, brain neoplasms

Introduction

Photodynamic therapy (PDT) of malignant tumors activates photoreactive molecules (photosensitizers) selectively taken up and retained by tumor cells. Porphyrins, especially hematoporphyrin derivatives (HpD), activated by red light have frequently been used. Earlier clinical trials have achieved encouraging results in cases of skin, breast, bladder, lung, and gastric malignancies. However, treatment of advanced large lesions is less satisfactory because the wavelengths used achieve limited tissue penetration. Furthermore, enhanced skin sensitivity to sunlight or strong artificial light is commonly associated with HpD.

Therefore, we have developed a new method of PDT using pheophorbide a (Ph-a) as the photosensitizing agent, which demonstrates reduced skin sensitization. We describe the encouraging results so far including higher concentration of Ph-a in brain tumors, deep tissue penetration by the neodymium yttrium-argon-garnet (Nd:YAG) laser, and efficacy in treating experimental brain tumors.

Materials and Methods

I. Photosensitizer

Ph-a was prepared from chlorophyll a using Endo’s method. Fluorescence spectroscopy showed the purity of Ph-a to be 70-90%. Solid Ph-a was dissolved in either albumin or phosphate-buffered saline (PBS) solution.

II. Distribution of Ph-a

Rat T9 glioma cells (1 × 10⁶/mm³) were inocu-
lated stereotactically into the thalamus of Fisher 344 (F344) rats (5-weeks-old, weight 120-140 gm). Ph-a (3.0 mg/kg) dissolved in albumin was administered intravenously 12 days after the tumor cell inoculation. The rats were sacrificed 24 hours after Ph-a injection. Samples of the tumor, brain, lungs, liver, spleen, kidneys, muscles, and skin were analyzed.

The tissue Ph-a concentration was determined by the following method. Tissue samples (less than 0.5 gm) were homogenized in a Polytron Homogenizer (Kinemica, Luzern, Switzerland) operated at full speed for 1 minute with 85% acetone (5.0 ml) containing 25 mg of hematoxylin (Hpp) solution (0.25 mg/ml dimethyl-sulphoxide) as an internal standard. The homogenate was centrifuged at 1000 G for 5 minutes. The supernatant was collected, mixed with 5% sodium sulfate solution (15 ml) and ethyl acetate (20 ml) in a separating funnel, and shaken vigorously. The upper layer was collected, washed twice with 5% sodium sulfate and dried overnight over 1.5 gm of anhydrous sodium sulfate. The solvent was then distilled under vacuum from the dried ethyl acetate extract (about 10 ml). The residue was dissolved in ethyl acetate (1.0 ml) and analyzed by high performance liquid chromatography (HPLC).

The fixed phase was a 3.9 mm x 30 cm column prepacked with 10.0 um C-18 particles (Bondapak, Waters Associate, Milford, Massachusetts, U.S.A.) and the mobile phase was a mixture of acetonitrile, tetrahydrofuran, 9 mM citric acid, and 7 mM dibasic sodium phosphate (6:2:1:1 by volume, pH 3.2). The flow rate was 1.0 ml/min at room temperature. The excitation and emission wavelengths used by the detector were 400 and 626 nm for Hpp, and 415 and 670 nm for Ph-a.

III. Skin photosensitivity due to Ph-a
Enhanced skin photosensitivity due to Ph-a was studied using F344 rats. Ph-a (30 mg/kg) dissolved in albumin was injected intravenously into rats. After 24 hours in darkness, the rats were exposed to 20,000 lux irradiation from a tungsten lamp for 2 hours.

IV. Laser
A continuous wave Nd:YAG laser (CL.50, wavelength 1063 nm, Surgical Laser Technologies Japan Co., Ltd., Tokyo) with a pulse generator (acoustic Q switch) and 600 um core quartz fiber delivery system were used.

The power density profile of the Nd:YAG laser was obtained in the rat brain for the continuous wave (0.6 W) and Q switched wave (0.6 W) using a fiber detector and a power meter (Model 815, Newport Corporation, Fountain Valley, Calif., U.S.A.) (Fig. 1).

V. In vitro study
The in vitro study included both treatment and control groups. The control group assessed the possible cytotoxic effect of the medium, PBS, and Ph-a in PBS. The thermal effect was studied at 44.0°C (waterbath hyperthermia).

Cultured rat T9 glioma cells (1 x 10^6) were cultivated for 12 hours in a flask with 1.0 ml growth medium (RPMI 1640, 10% fetal calf serum). The bottom of a flask measured 0.784 cm^2. Rat T9 glioma cells grew in a single layer. If required, the cells were incubated in PBS or Ph-a in PBS (10^-3 molar) solution for 3 hours and the solution removed before laser irradiation.

The flask was placed in a temperature controlled waterbath and the bottom irradiated for 20 minutes with the laser at an average power density of 3 W at the fiber tip. The peak pulse power was 93 kW and the pulse rate was 200 Hz. The tip of the quartz fiber was 10 cm away from the bottom of the flask. Two hours after irradiation, the cells were stained with trypan blue and the surviving tumor cells counted.

VI. In vivo study
Rat T9 glioma cells (1 x 10^6) were subcutaneously inoculated into the back of F344 rats (5-weeks-old, weight 120-140 gm). Twelve days later Ph-a (30 mg/kg) was injected either intraperitoneally or intravenously. The rats were kept in the dark for 24-48 hours before PDT.

At treatment, all tumors were larger than 10 mm in diameter. The rats were anesthetized with intraperitoneal injection of pentobarbital (30 mg/kg). The laser was interstitially irradiated with a frosted...
probe for 15 minutes. The average power density was 2.7 W at the fiber tip and the peak pulse power was 112 kW with 500 Hz pulse rate. The temperature was monitored 7–10 mm from the center of the probe. The rats were randomly divided into the following 3 groups: Group A (PDT group), Ph-a intravenous injection and laser irradiation with the peripheral temperature below 40.0°C; Group B (PDT/laser hyperthermia group), Ph-a intraperitoneal injection and laser irradiation with the peripheral temperature at 42.0–43.0°C; Group C (control group), Ph-a intraperitoneal injection without laser irradiation.

The treatment effects were assessed by measuring the tumor size in a bipolar direction using calipers after 1, 2, 3, 4, 5, and 6 weeks. We performed statistical analysis using Student’s t-test.

Results

I. Tissue distribution of Ph-a

Table 1 shows the tissue distribution of Ph-a in F344 rats bearing rat T9 glioma 24 hours after intravenous injection of Ph-a. The Ph-a concentration was highest in the liver and lung, and moderate in the spleen. It was very low in the normal brain tissue, but significantly higher in the T9 glioma with the very high tumor to normal brain tissue concentration ratio of 7.5:1 (p < 0.01).

II. Enhanced skin sensitivity

The rats developed no skin reactions due to Ph-a.

Table 1 Tissue distribution of pheophorbide a (Ph-a) in T9 glioma bearing rats 24 hours after intravenous Ph-a injection (3 mg/kg)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of measurements</th>
<th>Ph-a volume in tissue (µg/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebrum affected hemisphere</td>
<td>5</td>
<td>0.0084 ± 0.0030</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>3</td>
<td>0.0071 ± 0.0042</td>
</tr>
<tr>
<td>Brain tumor (T9 glioma)</td>
<td>6</td>
<td>0.063 ± 0.023*</td>
</tr>
<tr>
<td></td>
<td>(tumor/normal tissue differential)</td>
<td>7.5×</td>
</tr>
<tr>
<td>Blood</td>
<td>3</td>
<td>0.031 ± 0.011</td>
</tr>
<tr>
<td>Lung</td>
<td>3</td>
<td>1.4 ± 0.71</td>
</tr>
<tr>
<td>Liver</td>
<td>3</td>
<td>1.3 ± 0.22</td>
</tr>
<tr>
<td>Kidney</td>
<td>3</td>
<td>0.098 ± 0.044</td>
</tr>
<tr>
<td>Spleen</td>
<td>3</td>
<td>0.47 ± 0.065</td>
</tr>
<tr>
<td>Pancreas</td>
<td>2</td>
<td>0.021</td>
</tr>
</tbody>
</table>

Values are means ± SD. *p < 0.01 vs. normal tissue by Student’s t-test.

III. Power density profile

Both continuous and acoustic Q switched Nd:YAG laser pulses demonstrated even scattering of the laser beam near the surface of the contact probe, and penetrated deeply into the brain tissue (Fig. 2).

IV. In vitro PDT study

Table 2 shows the survival rates of rat T9 glioma cells cultured in vitro. The survival rate of Ph-a treated rat T9 glioma cells was 68.8 ± 5.4% after pulsed Nd:YAG laser irradiation for 20 minutes. This was significantly lower than in the control groups; 95.1 ± 1.6% for laser irradiation alone, 93.9 ± 3.9% for Ph-a treatment alone, 96.1 ± 3.4% for PBS treatment alone, and 98.2 ± 1.2% for medium alone (p < 0.01). This indicates that Ph-a was activated by a pulsed (acoustic Q switch) Nd:YAG laser causing the photodynamic effect. The survival rate was further reduced to 15.8 ± 3.3% after pulsed Nd:YAG laser irradiation in a waterbath at 44.0°C (p < 0.01). This indicates that the thermal effect enhanced the PDT induced cell damage. The

Fig. 2 Two-dimensional equi-power density profile of the Nd:YAG laser for continuous waves and acoustic Q switched waves. Irradiation was from a 5 mm frosted probe at 0.6 W and the power density was measured in the surrounding brain tissue.
survival rates in the control group were 57.2 ± 4.5% after laser irradiation for 20 minutes and 96.2 ± 3.2% for medium alone at 44.0°C (p < 0.01). This shows that the effect of laser irradiation increases at 44.0°C. There was no statistical difference in the control group, survival rates at 37.0°C of 95.1 ± 1.6% after laser irradiation for 20 minutes and 98.2 ± 1.2% without laser irradiation. This suggests that the efficacy of laser irradiation at 44.0°C was probably due to laser induced hyperthermia rather than the increased temperature.

V. In vivo study

Figure 3 shows the effect of in vivo tumor treatment. Tumors disappeared in four of six rats treated by intraperitoneal Ph-a injection and laser irradiation with the peripheral temperature below 40.0°C (Group A). Tumors disappeared in all six rats treated by intravenous injection of Ph-a and laser irradiation with the peripheral temperature at 42.0-43.0°C (Group B). The tumors grew without laser irradiation treatment (Group C). The results suggest that the tumors were effectively destroyed by PDT using the pulsed Nd:YAG laser and Ph-a, and the effect of PDT was enhanced by laser hyperthermia.

Discussion

The ideal photosensitizer for PDT should be nontoxic and selectively absorbed by malignant tissue. Furthermore, the activation wavelength should have deep tissue penetration. This study employed a new photosensitizing agent, a degradation product of chlorophyll a formed by removing the phytol group and magnesium ion (Fig. 4). Some chlorophyll degradation products cause photosensitivity reactions in sheep and cattle, and a certain brand of chlorella tablets are known to cause photosensitive dermatitis in man. Endo et al. studied the photosensitizing effect of Ph-a in rats and reported that a small amount (4.7 mg/kg/day) orally administered for 90 days caused no skin reactions. Chlorophyllases, which convert chlorophyll to pheophorbide, are widely distributed in plant tissue. Furthermore, pheophorbides are gradually produced in highly salted Japanese vegetable products.
ducts such as “Nozawana” and “Takanazuke.” However, the ingestion of a small amount of pheophorbides, including Ph-a, produced no skin reactions or other side effects in man.

This study showed that the moderate amount of Ph-a (30 mg/kg) necessary for PDT did not cause skin reactions or other side effects in rats exposed to a bright tungsten lamp when given intravenously. This suggests that Ph-a is a less toxic photosensitizing agent.

Maeda et al. reported that Ph-a is selectively accumulated in malignant tumors. In this study, the Ph-a distribution was analyzed by direct extraction from tissue samples and HPLC. The Ph-a concentrations were 63 ± 23 ng/gm in tumor tissue and 8.4 ± 3 ng/gm in normal brain tissue, 24 hours after the intravenous injection (3.0 mg/kg). Ph-a thus achieves a high tumor to normal brain tissue concentration ratio of 7.5:1 (p < 0.01).

For conventional PDT, the optimum wavelength is 630 nm obtained from either an argon dye laser or a pulsed gold vapor laser. These lasers are large and expensive, and the laser beams achieve limited tissue penetration which is inadequate for large tumors. The power density profile study revealed that the Nd:YAG laser penetrated deeply into brain tissue. The Nd:YAG laser power can be obtained from 0.1 W to 100 W from the equipment used for both conventional and endoscopic surgery.

Mashiko et al. studied the absorption and fluorescence spectra of Ph-a dissolved in PBS using a Q switched pulsed Nd:YAG laser (giant pulse). They observed that the fluorescence excited by the pulse increased with the square of the Nd:YAG laser intensity. Based on this quadratic intensity dependence, they concluded that the excitation of Ph-a by Q-switched pulsed Nd:YAG laser irradiation was a two photon absorption process. However, the giant pulsed Nd:YAG laser cannot be transmitted through a quartz fiber. We therefore used a continuous wave Nd:YAG laser equipped with the acoustic Q switch.

The in vitro study assessed effects of Ph-a and laser irradiation using a pulsed (acoustic Q switched) Nd:YAG laser. The results suggest that PDT significantly reduced the glioma cell survival rate and that this effect was probably due to the photodynamic effect. It also showed that hyperthermia enhanced PDT cell damage demonstrating the useful combination of PDT and laser hyperthermia. In the in vivo study, the tumors were eradicated in four of six rats by PDT and in all six rats by the combination of PDT and laser hyperthermia. Those tumors not responding to PDT were excessively large with contiguous metastatic deposits. Though the central parts of these tumors became necrotic after treatment, the tumor regrew from the periphery. Probably the laser intensity was inadequate at the tumor periphery to achieve complete cell destruction.

Hyperthermia has many advantages in treating cancers. It is effective against relatively radio-resistant hypoxic cells in the S-phase and poorly vascularized tissues which are resistant to most chemotherapeutic agents. The biological effect of hyperthermia depends on the duration and intensity. It has no cumulative toxicity and potentiates the effects of both chemotherapy and ionizing radiation. Our experiments show that laser hyperthermia also potentiated the effect of PDT. The Nd:YAG laser causes local hyperthermia. Suzuki et al. reported a computer controlled Nd:YAG laser system for local interstitial hyperthermia under rigid temperature control. The Nd:YAG laser can be transmitted through a flexible quartz fibers of 100-600 μm diameter which can easily be inserted into deep-seated brain tumors under stereotactic control.

The mechanism of PDT is still not clearly understood, but those proposed include singlet oxygen, hydroxyl radicals, membrane sensitization, vascular damage, and thermal effects. The capacity of PDT to produce tissue necrosis may be determined by both photodynamic and thermal effects. Therefore, a combination of PDT and hyperthermia in treating cancers is desirable. The in vitro study indicates the excellent results obtainable by a combination of PDT and laser hyperthermia with a single laser delivery system.

Acknowledgments

The authors thank Dr. Jun Yoshida, Department of Neurosurgery, Nagoya University School of

Neurol Med Chir (Tokyo) 31, May, 1991
References


Address reprint requests to: I. Fujishima, M.D., Department of Neurosurgery, Seirei Mikatabara General Hospital, 3453 Mikatabara-cho, Hamamatsu, Shizuoka 433, Japan.