Acute microvascular response to photodynamic therapy with mono-L-aspartyl chlorin e6 and a diode laser: Observation under modified operation microscope

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SUMMARY

Microvasculature change has been recently identified as an important factor of photodynamic therapy (PDT). Three experiments were performed to investigate this phenomenon. Two groups of mice bearing Meth-A tumor were treated with 664 nm light (100 J/cm², 100 mW/cm²). Five (group 1) or 20.0 mg/kg (group 2) of mono-L-aspartyl chlorin e6 (NPe6) was administered to these mice 2 or 24 hours before light exposure, respectively. Tumor response was more pronounced in group 1 (tumor cure ratio was 80% vs. 25%, p < 0.05). The concentration of photosensitizer in tumors or in plasma was measured by HPLC 2 hours and 24 hours after administration of NPe6. The values of the NPe6 in tumor were similar in both groups, however the level in plasma in group 2 was below threshold level as opposed to 2.33 ± 0.97 µg/ml in group 1. Tumor specimens obtained after PDT were examined by phosphotungstic acid hematoxylin (PTAH) staining. Some positive areas were detected in tumor vessels. All hemokinetic changes during or after PDT could be observed continuously using the improved microscope, which has a special notch filter preventing the passage of emission laser light. Microvascular changes were dominated by the appearance of emboli, which seemed to be platelet aggregation containing fibrin.

INTRODUCTION

Photodynamic therapy (PDT) is a useful treatment for solid local neoplasms as attested by beneficial results obtained in skin, bronchial, bladder, gynecological and other tumors¹⁻⁴. Especially in bronchial and bladder tumors, reports have described not only palliation of advanced tumors but also curative treatment of early stage cancer.

It has been suggested that the mechanisms of PDT can be divided into two categories⁵. One is direct cytotoxic effect. This denotes the selective destruction of cancer cells by reactive oxygen, especially singlet oxygen, induced by the photochemical reaction between a photosensitizer retained in malignant tissue and absorbed light. The electron microscopy investigation showed that initial changes occurred in mitochondria.
then endoplasmic reticulum and free ribosome, prior to nuclear changes of clumping of chromatin at the nuclear membrane. On the other hand, PDT-induced microvascular stasis effect results in secondary changes of the blood circulation through the tumor vessels. Several investigators have suggested that the changes in the microvasculature during and/or after PDT may be more important for the effectiveness of PDT than the direct toxicity based on a selective localization of photosensitizer. Further quantitative analysis of structure and/or function parameters is difficult with a complex mixture of porphyrins contained in Photofrin, which is the photosensitizer currently used in clinics, because of its unclear molecular weight. Recently, NPe6, a second generation photosensitizer, has received much attention as a promising new photosensitizer for use in PDT. Early stage clinical trials with NPe6 are now in progress.

The current study was designed to observe and demonstrate the microvascular effect continuously during the photoactivation of NPe6 in vivo.

**MATERIALS AND METHODS**

*Animals and tumor.* Female Balb/c mice, 4 weeks of age weighing about 16 g were supplied by Sankyo Laboratories, Tokyo, Japan. The mice were housed in animal quarters with controlled temperature and light, and fed chlorophyll-free mouse chow and water ad libitum. Meth-A fibrosarcoma was grown as a subcutaneous tumor in the right leg region implanted by injection of $1 \times 10^6$ tumor cells. About 1 week after implantation, the tumor volume reached around 0.1 cm³.

*Chemicals.* Mono-L-aspartyl chlorin e6 (NPe6) was supplied by Meiji Seika Kaisha Ltd., Tokyo, Japan. It was dissolved in saline at concentrations ranging from 0.1 to 20.0 mg/ml.

[Study 1] *In vivo treatment of mice with photosensitizer and laser.* Mice were injected intravenously with doses of 5.0 mg/kg or 20.0 mg/kg of NPe6. Laser irradiation was performed at 2 and 24 hours after the administration of NPe6 (Table 1). These PDT conditions were determined by preliminary experiment in our institute (data not shown). The light source was a diode laser system set at 664 nm wavelength, supplied by Panasonic Ltd., Osaka, Japan. The output power of the laser beam was set to achieve 100 mW/cm² and irradiated to deliver a dose of 100 J/cm². The diameter of the irradiation area was 14 mm, broad enough to cover the entire tumor. One week after PDT, the mice were sacrificed and the tumor response was evaluated by histopathological examination. Tumor response criteria were based on the percentage of complete remission in each group.

*High Performance Liquid Chromatography (HPLC) analysis.* Concentrations of NPe6 in plasma and implanted tumor were analyzed using HPLC. Samples of plasma and tumor were taken from mice in two groups, 2 and 24 hours after NPe6 administration with doses of 5.0 mg/kg or 20.0 mg/kg intravenously, respectively. These were the same conditions as described above for group 1 and 2 just before laser exposure. Sample preparations were performed as shown in Fig. 1.

[Study 2] *Observation of vascular effects.* We used a modified operation microscope (Microphoto-FX type, Nikon, Tokyo, Japan) to observe microvascular changes on PDT in vivo. After removal of abdominal hair, the abdominal skin of the mouse was incised and a flap was made with as little injury to vessels as possible, and the mouse was immobilized to the stage of the microscope. A super notch filter, supplied by Kaiser Optical System Inc., Michigan, USA, that prevents passage of 662 ± 2 nm wavelength light, was attached to this device in front of the CCD camera (Storage Camera Moder CI-11 and Moder CC-200, Visitec, Grevesmuhlen, Germany) (Fig. 2). The photosensitizer was injected intravenously at doses of 1.0, 2.0, and 5.0 mg/kg while mice were already fixed to the stage. Laser irradiation was performed immediately after the photosensitizer

<table>
<thead>
<tr>
<th>Group</th>
<th>NPe6 dosage (mg/kg)</th>
<th>Time (hours)</th>
<th>PDT conditions (W/cm², J/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>

NPe6 was administered intravenously.

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**Table 1** PDT conditions of groups 1 and 2 in Study 1.
Tumor tissue 0.15 g

- added 0.15 ml of 0.4% EDTA/50 mM HEPES, pH 7.4
- homogenized
- added 5.0 ml of HClO4 : CH3OH = 1 : 1
- homogenized
- added 20 ml Distilled water
- centrifuged at 3000 r.p.m. for 15 min. at 5°C

Supernate 1 → Ppt. 1 → repeat * step
Supernate 2 → Ppt. 2

SEP-PAK IC18 eluted 2 ml of CH3OH
Sample for HPLC

Plasma plasma 0.1 ml

- added 0.1 ml of 0.4% EDTA/50 mM HEPES, pH 7.4
- added 0.7 ml of CH3OH
- mixed by vortex mixer for 5 sec.
- centrifuged at 12000 r.p.m. for 5 min. at 5°C

Supernate → Ppt.
Sample for HPLC

**Fig. 1** Sample preparation for HPLC analysis

administration at a power of 5 mW/cm² for the required time, ranging from 30 to 60 min. The irradiation area was 5 mm in diameter to cover the entire microscopic view. The irradiation laser was oriented in the same direction as white light. Hemokinetic changes before, during and after PDT were recorded continuously using a computerized S-VHS video system. The entire process was done under general anesthesia by ether. We also observed the vessels in mice that received no photosensitizer, using either white light or laser irradiation for at least 1 hour, as controls.

[Study 3] Histological study. Phosphotungstic acid hematoxylin (PTAH) staining, which helps identification of fibrin, muscle striations, intercellular bridges, and cilia, was performed with tumor specimens treated with PDT. The dose of NPe6 was 5 mg/kg. Light treatment performed at 2 hours after NPe6 administration was 100 mW/cm² and 100 J/cm². Tumor specimens were taken immediately after PDT and fixed in formalin.

**RESULTS**

[Study 1] Two groups of 12 tumor-bearing mice were treated with PDT using NPe6 and the diode laser. Complete remission was obtained in 83.3% and 25% (in 10 and 3 mice in group 1 and group 2 respectively, p < 0.05, two proportions comparison test) (**Fig. 3**). Concentrations of NPe6 in plasma and tumor were analyzed by HPLC. In group 1, plasma and tumor samples were taken 2 hours after 5 mg/kg of NPe6 administration. In group 2, samples were taken 24 hours after 20 mg/kg of NPe6 administration. These dosage and time delay in both group 1 and 2 were the same conditions as used in the tumor response experiment above. The concentration of NPe6 in tumor was 1.18 ± 0.46 and 1.20 ± 0.47 µg/g tissue in
Two groups of 12 tumor-bearing mice were treated with PDT using NPe6 and a diode laser. In group 1, mice were irradiated 2 hours after 5 mg/kg of NPe6 administration. In group 2, mice were irradiated 24 hours after 20 mg/kg of NPe6 administration. The percent cure in group 1 was 83%. In group 2 it was 25%. Tumor responses to PDT in vivo were based on the percentage of complete remission in each group, as evaluated by histopathological examination at seven days after PDT. The difference in tumor response between the two groups was statistically significant (p < 0.05; two proportions comparison test).

However, the plasma concentration of NPe6 in group 1 was 2.3 ± 0.97 μg/ml while in group 2 it was below the level of HPLC detection (Fig. 4).

[Study 2] Abdominal subcutaneous veins were observed microscopically before, during and after PDT continuously. At different times after the onset of laser irradiation, which depended on the PDT dose used, white floating emboli appeared in the blood stream. The emboli subsequently attached to the vessel wall, and, in some cases, completely occluded blood flow. The onset of these hemokinetic changes depended on NPe6 dose. When the doses of NPe6 were 1.0, 2.0, 5.0 mg/kg, appearance times of white emboli were about 15, 3, 1 minutes, respectively (n = 2). Representative examples of microscopic images recorded by the video system are shown in Fig. 5 in a mouse given 1.0 mg/kg of NPe6 immediately before laser irradiation. The image A was recorded before photosensitizer injection and laser exposure, while B, C and D were taken during laser irradiation at 10, 20 and 30 minutes after the photosensitizer injection, respectively. The first hemokinetic changes can already be seen in B. The emboli appeared at the bifurcation of the vessel and interfered with the blood stream (indicated by s). The number of emboli and their attachment to the vessel wall increased with time of laser exposure. The inside diameter of the vessel in B and C progressively decreased but the outside vessel diameter in D was still the same as that in A (indicated by ▼). Irreversible blood flow stasis occurred approximately after 1 hour of laser exposure. In the control mouse, with room light without photosensitizer administration, no white emboli could be seen in the target area before PDT. In addition, no significant change could be seen during or after laser irradiation for at least one hour without photosensitizer administration either (data not shown).

[Study 3] A histological image of PDT treated Meth-A tumor is shown in (Fig. 6). Two positive areas for PTAH, suggestive of a fibrin-containing structure, are indicated by triangles. Two vessels can be seen on the right and left sides of this image. In the vein on the left side, a PTAH-positive area attached to the right side wall occupied
about one quarter of the lumen. In the vein on the right side, a PTAH-positive area was located in almost the center of the vessel. These areas were attached to the vessel walls.

DISCUSSION

Despite progress in clinical use, the real mechanism on which the PDT effect is based is still not completely clear. It was suggested that the changes in microvasculature during and/or after PDT are more important for the effectiveness of PDT than the direct cytotoxicity that is based on selective localization of photosensitizer in malignant tissue\(^{16,17}\). In study 1 presented in this work, tumor response to PDT in group 1 (5 mg/kg of NPe6 2 hours before light treatment) was more than three times greater than in group 2 (20 mg/kg of NPe6 24 hours before light treatment) (Fig. 3). The HPLC analysis demonstrated that tumor concentrations of NPe6 in both groups were almost at the same level, at about 1.2 \(\mu g/g\) tissue. The factor responsible for the difference between these two groups was obviously the concentration of photosensitizer in plasma at the time of light exposure (Fig. 4). The results of study 1 indicate that the amount of photosensitizer in plasma at the time of laser irradiation was more important for the effectiveness of PDT in vivo than that in the tumor. Therefore, it is important to investigate changes that occur inside tumor vessels during and after PDT.

Star et al and van Leengoed et al observed such hemokinetic changes by using a Sandwich observation chamber\(^{14,15}\). Reed et al studied vessel constrictions after PDT with red blood cell column diameter measured by a fluorescent microscope\(^{12,18,19}\). We were able to observe the
microcirculation changing phenomenon in vivo during PDT continuously using a modified operation microscope. The super notch filter (Fig. 2) avoids an interference by the laser beam blaze and enabled us to observe the phenomenon continuously during laser irradiation with a natural view under white light and in real time on the monitor. Since the CCD camera was connected to a video system with the computer, it was also possible to record the entire development of hemokinetic changes during the ongoing experiment and to process captured images using suitable software.

Hemokinetic changes appeared after a few minutes of laser irradiation. The most prominent was the appearance of white emboli floating within the vessels. Some of these emboli narrowed the diameter of blood stream in vessels and some of them attached to bifurcations of vessels, subsequently stopping the blood flow. These hemokinetic changes did not seem to be due to vessel constriction, since no change in vessel contours compared to the status prior to the light treatment was observed (Fig. 5). Some of the microcirculation stases seemed reversible under certain PDT conditions and the others were irreversible. McMahon et al also observed complete blood flow stasis in cremasteric vessels, but no vessel constriction in animals given PDT using NPe6[20]. Chaudhuri et al concluded that the tumor endothelial cell and endocapillary layer is rapidly destroyed during haematoporohyrin derivative based photodynamic therapy[21]. These vascular changes probably lead to tumor anoxia that may play a significant role in the destruction of PDT treated tumors[2]. Although our results indicate that the appearance of the emboli is dependent on PDT dose, more research is required to precisely define conditions such as photosensitizer dosage, light power density and time delay associated with this phenomenon.

The PTAH staining of the tumor section obtained from the tumor specimen taken immediately after PDT showed positive areas localized in vessel lumens attached to the vessel walls (Fig. 6). PTAH stains a great variety of structures, but is particularly useful for muscle striations, intercellular bridges, fibrin, and cilia. In the case of our tumor specimen, the positive areas were considered to identify fibrin because those positive areas were located in vessels and other structures were unlikely to be in the vessels except fibrin.

Fig. 6 The results of tumor staining after PDT
Tumor specimen was taken just after PDT and fixed by formalin. PTAH staining was performed. Two positive areas (▽) can be seen in veins. Original magnification was × 400.
The endogenous fibrin may be induced by the activation of a coagulation pathway. This result, in the context of our observations of PDT-treated normal vessels in study 2, suggests that the emboli that attached to vessel walls and stopped microcirculation were likely platelet aggregates including fibrin formations induced by the photochemical reaction. In our experimental model, the amount of accumulated NPe6 in endothelial cells of vessels could be ignored, because laser irradiation was performed immediately after photosensitizer administration, and it took less than 15 minutes to recognize hemokinetic changes.

These hemokinetic changes were likely to depend mainly upon the direct effects on platelets or other blood components. It was suggested that platelet aggregation leading to blood flow stasis might result from damage to the platelet itself rather than the endothelial cell. However, a complete lack of endothelial cell damage during PDT is unlikely, since fibrin formation was found near the inside wall of vessels. It is more likely that damage occurs to both platelets and endothelial cells, but the platelet response might be stronger. It was suggested that such response might be more relevant to therapeutic effects\(^1\). It was also suggested that vascular effects on PDT are multi-target phenomena with contributions from many components of vascular wall and blood\(^6\).

The microvasculature hemokinetics in the mechanism of PDT requires further study, as it may differ with various photosensitizers. Photofrin and NPe6 have different subcellular sites of localization, but both damage vascular targets \textit{in vivo}\(^1\). Therefore this kind of study is important and deserves intense further investigation. Using our improved microscope should assist clarifying how PDT-induced hemokinetic changes are produced and affect vascular shutdown.

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Mono-L-aspartyl chlorin e6 とダイオードレーザーを用いた
光線力学的治療法における急性期微小血流反応:
改良型手術顕微鏡による観察

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要旨：PDT における急性期微小血流反応は重要な作用機序の一つと考えられていた。これに対する基礎的実験及検討を行った。腫瘍内 NPe6 濃度が同程度の腫瘍移植マウス 2 群間の抗腫瘍効果は、血漿中 NPe6 濃度が HPLC の検出限界未満の群で有意に低かった。励起波長 664 nm の光に対する notch filter を設置した改良型手術顕微鏡を用いた正常マウス腹部皮下血管における PDT による急性期微小血流反応の経時的観察では、レーザー照射直後から粘性の高い浮遊物が血流中に認められ、血栓を形成し血流の停滞・途絶を惹起した。PDT 直後の腫瘍内微細血管中に PTAH 染色陽性部分が認められた。PDT による急性期微小血流反応はフィブリンを主構成成分とする血管内塞栓物質形成が主体であり、実験系における抗腫瘍効果の差を惹起したと考えられた。また改良型手術顕微鏡は、これらの微小血流動態のリアルタイムでの解析の一助となると考えられた。