Photoactivated Photofrin II: Astrocytic Swelling Precedes Endothelial Injury in Rat Brain

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Abstract. Light activation of circulating hematoporphyrin derivatives has been used in the treatment of selected brain tumors. The effects of this photodynamic therapy on the non-neoplastic, adjacent brain tissue are incompletely characterized. We studied in adult Fisher rats the time-dependent (1 hour to 7 days) effects of photoactivated Photofrin II. Our protocol was comparable to that used in the treatment of human brain tumors. Structural and functional changes spread from the treatment surface and from the center to the periphery to involve the entire cerebral cortex exposed under a 5 mm craniectomy. The sequential changes spreading from the surface to the deepest cortical layer involve first astrocytes (1 hour), then endothelial cells and, ultimately, neurons. Thrombi were first noted in the microvasculature after 18 hours and coagulation necrosis of the entire area at risk occurred only after 48 hours. The results suggest that the photosensitizing agent crosses the intact blood-brain barrier and enters the astrocytic compartment where it becomes cytotoxic upon light activation. A comparison between the focal brain lesions of photodynamic therapy and those induced by middle cerebral artery occlusion suggests that cell damage evolves along different paths in these two forms of brain injury.

Key Words: Astrocytic swelling; Blood-brain barrier; Brain necrosis; Endothelial degeneration; Histopathology; Photodynamic therapy; Rat.

INTRODUCTION

Circulating hematoporphyrin derivatives (HPD) are preferentially taken up or retained by malignant neoplastic tissues, as suggested by the red fluorescence observed with ultraviolet light (1, 2). Malignant tumor cells containing HPD can be selectively destroyed by activating the absorbed product with visible light (3, 4). These principles have been applied both to define the location and to promote necrosis of human and experimental neoplasms involving the skin (5), the urinary bladder mucosa (6, 7), and the brain (8–10). The effect of photosensitizers on normal brain is incompletely characterized; therefore, the time course of cellular changes induced in brain by photodynamic therapy needs to be defined. In normal animals several investigators have induced focal brain lesions with various photosensitizing substances, and the resulting lesions have been likened to a brain infarction (11–18). Utilizing regimens that are becoming standard in the treatment of human brain tumors, HPD have been reported not to penetrate brain areas having an intact blood-brain barrier (BBB) and not to damage the normal human brain (15, 16). In contrast, Berenbaum et al (19) and Stroop et al (14) report early endothelial injury and tissue necrosis of the mouse brain after photoactivating either HPD or Photofrin I through the intact skull.

The objectives of this study were to clarify and document the evolution of cell damage after treatment of brain with Photofrin II, and to compare the brain lesions of Photofrin II with those that characterize an early brain infarction. We adopted a protocol comparable to that used in the treatment of human brain tumors and conducted a time-dependent study of the brain lesion at intervals ranging from 1 hour (h) to 7 days after the activation of Photofrin II.

MATERIALS AND METHODS

Forty-eight adult male Fisher rats (average body weight: 225 g) were used in this study. One experimental group (n = 38) and two control groups (n = 10) were prepared as shown in Table I. Six control and all experimental rats were injected intraperitoneally (ip) with 12.5 mg/kg of sterile Photofrin II (Quadralogic Technologies Ltd, Vancouver, Canada), osmolality: 179 mosmol/kg and pH: 7.23. The control animals in group B (Table I) were prepared at a subsequent date to those in group A and the experimental group. Forty-eight hours after Photofrin II injection in those that received it, rats in all groups were given ip ketamine (44–80 mg/kg), xylazine (13 mg/kg) and atropine (0.04 mg/kg) in preparation for a craniectomy. The method employed to perform the craniectomy and the laser treatment are described in detail elsewhere (18). Briefly, the rat’s scalp was shaved and a midline incision was made; a craniectomy, 3 mm in diameter, was made from the midline extending over the right hemisphere posterior to the coronal suture; the laser beam was shone over the intact dura mater for a period of 3.44 minutes (min). The control rats in group A were not exposed to the laser beam, and animals in group B were exposed to laser but received no PhotofrinII. Core temperature was maintained (37°C) via a recirculating pad and K module and monitored via an intracranial type T thermocouple. An argon-pumped dye laser (Coherent Radiation, Palo Alto, CA) was utilized at wavelength of 632 ± 2 nm. The fiber optic was placed approximately 10 mm from
Fig. 1. The brain microvasculature in PDT lesions, after iv HRP injection. Vibratome sections of 100 µm thickness. A-E: ×13; F: ×26. A. One h after PDT. Normal microvasculature in the exposed area. V, lateral ventricle. B. Four h. Multifocal leakage of HRP in the cerebral cortex. C. Twelve h. Diffuse leakage of HRP in the cortex. D. Eighteen h. Localized leakage of HRP in the surrounding cortex, white matter, and hippocampus; abnormal microvasculature in the lesion. E. Forty eight h. Some blood vessels with enlarged diameter are visible, but most microvessels are thrombosed, leaving a "blank" area free of HRP (arrows). F. Seven days. Coagulation necrosis and early cavitation (asterisks) in the lesion. Regenerating blood vessels at the margin of this lesion are invisible.

The dural surface of the brain. The optical power density onto the dural surface was maintained at 100 mW/cm². Brain temperature was monitored during exposure of the laser with a thermocouple inserted in the brain to a depth of 1 mm at the periphery of the craniectomy site. All animals were treated with 35 J/cm² of optical energy. After exposing the dura to the laser, the scalp was suture closed. The animals were allowed to recover and were monitored for several hours following surgery.

Two tracers with different molecular weights were used to assess BBB damage caused by the photodynamic therapy (PDT): Evans blue (EB, 960 daltons as the native molecule and approximately 69 kilodaltons when conjugated with serum albumin, Sigma Chemical Co., St. Louis, MO) and horseradish peroxidase (HRP, type VI, 43 kilodaltons, Sigma). All rats were injected intravenously (iv) with 1 ml of a 2% Evans blue solution 30 min before death. Fifteen min before sacrifice, one or two rats from each experimental group were also injected iv with 50 µg/g of HRP, freshly dissolved in 0.9% sodium chloride solution.

Four to seven rats were killed at each time point (Table 1). Under general anesthesia, each rat was transcardially perfused with either 4% paraformaldehyde (FF) in 0.1 M phosphate buffer (PB) for routine histological and histochemical studies, or with 3% glutaraldehyde (GA) in 0.1 M PB for electron microscopic study. The brains fixed in 4% FF were removed, photographed and immersed overnight in fixative at 4°C. Following fixation, the brains were coronally cut into slices of 1.5-2.0 mm thickness. The slices including the area irradiated with the laser were embedded in paraffin. One of the coronal slices containing the lesion was immersed in 3% GA and used for electron microscopic examination as described below. The brains that were perfusion fixed with 3% GA were cut into slices 1 mm thick and further trimmed into sample pieces (1 × 1 × 2 mm). These samples were post-fixed in 1% osmium tetroxide for 3 h, dehydrated through graded ethanols and embedded in araldite. Semithin sections (approximately 1 µm thick) were stained with toluidine blue (Fig. 2), and ultrathin sections of the area of interest were prepared and examined with a Philips 300 electron
### TABLE 1

Treatment Protocol

<table>
<thead>
<tr>
<th>Time after</th>
<th>Number of rats</th>
<th>Fixation procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>craniectomy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Group A</td>
<td>1 h</td>
<td>3</td>
</tr>
<tr>
<td>P-II#, no laser</td>
<td>24 h</td>
<td>3</td>
</tr>
<tr>
<td>Control Group B</td>
<td>1 h</td>
<td>1</td>
</tr>
<tr>
<td>laser, no P-II#</td>
<td>24 h</td>
<td>1</td>
</tr>
<tr>
<td>48 h</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7 days</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Experimental Group</td>
<td>1 h</td>
<td>4</td>
</tr>
<tr>
<td>P-II# and laser</td>
<td>4 h</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>18 h</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>5</td>
</tr>
<tr>
<td></td>
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<td>5</td>
</tr>
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<td></td>
<td>7 days</td>
<td>4</td>
</tr>
<tr>
<td>Totals</td>
<td>48</td>
<td>22</td>
</tr>
</tbody>
</table>

* PF: Perfusion fixation with 4% paraformaldehyde.
** HRP-PF: Immersion fixation in 4% paraformaldehyde after Evans blue and horseradish peroxidase injections.
*** GA: Perfusion fixation with 3% glutaraldehyde.
# P-II: Photofrin II ip injection (12.5 mg/kg).

Results

Control animals treated either with Photofrin II or with laser irradiation alone were sacrificed at various time intervals after the craniectomy (Table 1); their brains showed no significant abnormality in structural or tracer studies, except for minimal neuronal shrinkage and astrocytic swelling attributed to the operative procedures and the insertion of the thermocouple. These results confirm previous observations made on 46 Fisher rats divided as follows: group I: craniectomy only, n = 16; group II: craniectomy, Photofrin II and no laser, n = 16; and group III: no Photofrin II and laser exposure 140 J/cm², n = 14. None of these animals showed histological evidence of brain damage at 4, 24, 72 h and 7 days after the treatment (18). In the current experimental group, the histological features of the contralateral cerebral hemisphere were identical to those seen in the two control groups. Mean body core temperature: 36.1°C, SD ± 0.54; mean brain temperature: 34.8°C, SD ± 0.88.

One Hour after PDT: Grossly, there was minimal leakage of EB and HRP confined to the subarachnoid space at the center of the site of laser irradiation. The subarachnoid space was not completely filled by the dye, suggesting some degree of shrinkage of the brain. However, the dye did not extend beyond the site of irradiation. Histologically, there was no evidence of brain damage at this time. Photofrin II - Photochemotherapic Effects in Rats.
Fig. 3. PDT lesion after 1 h. A. A swollen astrocyte with intact organelles and visible intermediate filaments (arrow). ×4,600. B. Swollen perivascular astrocytic process (A); the circumference of the vascular lumen (L) is deformed. Neuron (N) and synaptic terminals (arrows) are normal. ×12,600. C. A few perineuronal astrocytic processes (A) are swollen, but the intrinsic features of the adjacent neuron remain intact. ×12,200.
noid vessels were free of thrombi but scattered endothelial cells in the subarachnoid space showed aggregation of electrondense materials; endothelial gaps and leakage of amorphous materials were also noted. There was no parenchymal leakage of macromolecules (Fig. 1A); however, vacuolation of the neuropil and swelling of astrocytic nuclei were noted in the superficial cortical layers (Fig. 2A, B). Astrocytes in these areas were enlarged both in the perikaryon and cell processes (Fig. 3A–C). Capillary lumina were deformed (lost their circular profile) and perivascular swollen astrocytic processes were visible. Endothelial cells, basal lamina and pericytes were identical to those visible in the control animals and in the contralateral hemisphere. Scattered neurons in cortical layers II and III appeared scalloped but neuronal nuclei and cell organelles were well preserved. In the deep cortical layers, neurons were normal while astrocytes were swollen.

Four Hours after PDT: There was multifocal leakage of EB and HRP (Fig. 1B). Horseradish peroxidase was prominently visible around large blood vessels. Diffuse sponginess of neuropil was more obvious than before from the molecular layer to layers IV and V. Swelling of astrocytic nuclei and cell processes, and the accompanying luminal deformity of most microvessels (Fig. 4), were prominent. Capillary endothelial cells still appeared normal. Scattered neurons showed dilation of the Golgi apparatus and the rough endoplasmic reticulum cisternae; however, the nuclear and dendritic components of these cells were normal.

Six and Twelve Hours after PDT: There was diffuse and massive leakage of EB in the entire irradiated brain area (Fig. 1C). The cerebral cortex lesions spread to layer VI. Other histological features at these two time points (6 and 12 h) were very similar. In the superficial layers (I–IV), scalloped neurons and clear, swollen astrocytes were widely distributed; the neuropil appeared spongy (Fig. 2C). The lumina of most small blood vessels in the lesion contained red blood cells (Fig. 5A, B). A few granulocytes were visible in the neuropil and in the perivascular spaces in the superficial cortical layers. The endothelial lining in capillaries and veins (venules) often showed gaps and basal lamina exposure (Fig. 6). At the sites of denudation, platelets adhered to the basal lamina. The walls of capillaries containing granulocytes appeared thinned and denuded. Horseradish peroxidase became visible within the endothelial cytoplasm (Fig. 5A), in the basal lamina (Fig. 5B), and in the extracellular space. Petechiae were noted for the first time at the marginal zone of the lesion.

Eighteen to Twenty-four Hours after PDT: Extravasation of HRP and EB was confined to the marginal zone of the lesion (Fig. 2D). In the center, a small number of microvessels showed HRP in their lumina; the remaining vessels had a rigid appearance and frequently showed HRP leakage. The cortical lesion was well demarcated from the surrounding cortex and showed spongy neuropil; plasma membranes of most neurons were disrupted and cell organelles became vacuolated (Fig. 7B). Many small blood vessels contained red blood cells, platelets and fi-
brin; endothelial cell nuclei were pyknotic (Fig. 7A). The swollen astrocytes also showed degenerative changes (Fig. 7C). Infiltration by granulocytes, lymphocytes and monocytes increased at this time.

Forty-eight Hours to Seven Days after PDT: The leakage of EB was confined to a portion of the lesion's marginal zone. The number of microvessels with intraluminal HRP markedly decreased, and only some large penetrating vessels contained HRP (Fig. 1E, F). The features of coagulation necrosis (e.g. the stainability of the nucleic acids disappeared in almost all cells) became apparent (Fig. 2D). A few granulocytes, lymphocytes, and macrophages were visible in the marginal zone. Lipid-laden macrophages were visible at the interface between the lesion and the surrounding brain. The evolution of the PDT lesion is summarized in Table 2.

DISCUSSION

Clinical trials among brain tumor patients have used Photofrin II as a photosensitizer utilizing the following combinations: drug dose at 3–5 mg/kg (8, 15, 16, 21, 22), light energy dose ranging from 1 to 230 J/cm² (23, 24), and time intervals between drug injection and photoactivation of 6–72 h (16, 22). Optimal treatment parameters have yet to be established (22). We chose a dose of 12.5 mg/kg in the rat as one that gives the most reproducible results (18, 25–27).

Several types of chemicals including rose bengal and porphyrin derivatives have photosensitizing properties; the former, a member of the xanthine family, is the most efficient generator of singlet molecular oxygen (28). Circulating rose bengal induces marked endothelial injury and thrombosis immediately after photoactivation (11, 29–31). Therefore, the application of rose bengal would not be suitable to the treatment of human brain tumors.

Substances in the hematoporphyrin group, including HPD, Photofrin I and Photofrin II, have been used in human and animals both to detect neoplasms and as an adjunct in the therapy of brain tumors (2, 5, 23). Accumulation of hematoporphyrins in gliomas is thought to

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Fig. 6. Twelve h after PDT. Vein in the subarachnoid space showing endothelial denudation (arrows) and platelet (P) adhesion to the exposed basal lamina. ×11,300.

<table>
<thead>
<tr>
<th>Time after craniectomy</th>
<th>Number of rats</th>
<th>Neurons</th>
<th>Astrocytes</th>
<th>Microvasculature</th>
<th>Leakage of macro-molecules</th>
<th>Microvascular circulation: HRP study</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1 h 3</td>
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<td>None</td>
<td>None</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>24 h 3</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>Control Group B</td>
<td>1 h 1</td>
<td>Minimal shrinkage: layers II, III</td>
<td>Minimal shrinkage: layers II, III</td>
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<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>24 h 1</td>
<td>Minimal shrinkage: layers II, III</td>
<td>Minimal shrinkage: layers II, III</td>
<td>None</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>48 h 1</td>
<td>Minimal shrinkage: layers II, III</td>
<td>Minimal shrinkage: layers II, III</td>
<td>None</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>7 days 1</td>
<td>Minimal shrinkage: layers II, III</td>
<td>Minimal shrinkage: layers II, III</td>
<td>None</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>Experimental Group</td>
<td>1 h 4</td>
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<td>+</td>
<td>Swelling +</td>
<td>Multi-focal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>4 h 7</td>
<td>None</td>
<td>+</td>
<td>Swelling +++</td>
<td>Multi-focal</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>6 h 4</td>
<td>+</td>
<td>Swelling +++</td>
<td>Deformed lumina+</td>
<td>No leak in parenchyma</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>12 h 5</td>
<td>+</td>
<td>Swelling +++</td>
<td>Deformed lumina+</td>
<td>Diffuse</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>18 h 4</td>
<td>Eosinophilia ++</td>
<td>Breakdown</td>
<td>Thrombi ED*</td>
<td>Diffuse</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>24 h 5</td>
<td>Eosinophilia ++</td>
<td>Breakdown</td>
<td>Thrombi ED*</td>
<td>Only at margin</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>48 h 5</td>
<td>CN**</td>
<td>CN**</td>
<td>CN**</td>
<td>CN**</td>
<td>No flow</td>
</tr>
<tr>
<td></td>
<td>7 days 4</td>
<td>CN**</td>
<td>CN**</td>
<td>CN**</td>
<td>No</td>
<td>No flow</td>
</tr>
</tbody>
</table>

* ED: endothelial degeneration; ** CN: coagulation necrosis.
Fig. 7. Eighteen h after PDT. A. A small parenchymal blood vessel shows endothelial (E) degeneration with nuclear chromatin clumping and cytoplasmic disorganization; a thrombus made of platelets (P) and fibrin (F) fills the lumen. Fibrin is also visible in the extracellular space. ×9,900. B. A degenerating neuron with nuclear chromatin clumping; organelles are segregated. ×6,600. C. A swollen astrocyte shows early degenerating features including nuclear chromatin clumping; the cytoplasm contains a small number of vacuolated organelles and intact mitochondria. ×6,300.
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depend on an impaired BBB, typical of malignant gliomas. Based on the absence of fluorescence in the brain, Wise and Taxdall (32) had concluded that circulating hematoporphyrins do not cross the intact BBB, and photosensitizing effects on the brain tissue around a tumor were not expected. Evensen et al (33) identified by chromatographic methods low-polarity compounds which are the most effective photosensitizers in Photofrin II and are separable from the fluorescent compounds. This may account for reports of definite brain damage in normal experimental animals after PDT (9, 13, 18, 25, 34). Several experiments revealed neuronal and endothelial damage with eventual coagulation necrosis; these lesions were thought to be identical to a cerebral infarct (9, 14, 18, 25). Berenbaum et al (19) and Strop et al (14) observed perivascular astrocytic swelling during the early stages of the lesion; these authors interpreted such change as a reflection of abnormalities in endothelial cell permeability.

In our experiments the astrocytic swelling occurred before the endothelial cell became permeable to macromolecules; also, the astrocytic swelling spread from the surface to the deeper cortical layers over a period of hours. This suggests that astrocytic swelling is the primary change induced by PDT. Since the porphyrins (Photofrin II) had been given 48 h before the brain was exposed to light, we assume that Photofrin II had initially entered the normal astrocytes. Chemical analysis of HPD incorporation into the normal brain tissue by either fluorescence or by means of isotope-labeled HPD or 3H-tetacycline demonstrates that hematoporphyrin derivatives do cross the intact BBB for as long as 72 h after the iv injection (9, 21, 34). Thus, the initial tissue changes in the PDT lesion may be induced by direct photodeactivation of Photofrin II within the astrocytes leading to the subsequent formation of cytotoxic free radicals within the astrocytic compartment.

The extent of brain damage increases with increasing laser energy under conditions of fixed HPD dose (25). When the laser energy remains the same, the extent of tissue damage increases as HPD concentration rises (9). In our experiments, the damage remained confined to the area located beneath the craniotomy, indicating that tissue changes depend on direct exposure to light. Laser exposure of the dural surface (in the absence of HPD administration) is said to produce brain necrosis under the dura (35); however, the effects of heat were not discussed in that experiment in which the energy (817 J/cm²) was considerably higher than that used in humans or that used in experiments (35 J/cm²).

There are also significant differences in the experimental design of the work by Strop et al (14). The photosensitizer (Photofrin I), the dose (25 mg/kg), the optical energy (200 mW) and the time of HPD injection (4 h before sacrifice) are all different from those used. In our study, endothelial damage occurred mainly in the microvasculature but this injury became apparent only after marked swelling and cytoplasmic membrane injury of astrocytes (1–4 h) had spread throughout the entire cortex at risk; this was associated later with intravascular thrombosis (12–24 h). These vascular changes were followed by coagulation necrosis of neurons, glia and blood vessels (48 h–7 days).

The histopathological features of the PDT lesion contrast with those of a focal ischemic lesion. In brain lesions induced by an arterial occlusion, the initial change involves neurons, while associated abnormalities occur in scattered glial and endothelial cells (36–38). Prominent histologic features of focal ischemic lesions include the heterogeneous and multifocal nature of the changes. This pattern of injury is attributed to multiple factors, including efficiency of the collateral circulation, magnitude and duration of changes in blood flow, vulnerability of individual neurons, changes in brain temperature, and several others (36, 39, 40). Support for a different mechanism and evolution in tissue damage between a PDT lesion and a focal brain ischemic lesion derives from nuclear magnetic resonance studies. Changes in brain energy metabolism, e.g. the ratio of β-adenosine triphosphate to inorganic phosphate, and intracellular pH, within the PDT lesion, suggest that there is direct cellular injury (probably astrocytic) in concert with secondary vascular thrombosis (26). Nuclear magnetic resonance imaging analysis based on the nature of changes in T-1 and T-2 relaxation times and water proton diffusion coefficient indicate that these parameters change in a manner different from that observed after occlusion of a middle cerebral artery (27). As suggested by Perria et al (24) and Wharen et al (22), our increased understanding of the PDT effects on non-neoplastic brain tissue will enlarge the numbers of applications that photosensitizing agents may have in the treatment of human brain tumors.

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