Distribution of hematoporphyrin derivative in the rat 9L gliosarcoma brain tumor analyzed by digital video fluorescence microscopy

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A digital video fluorescence microscope technique was used to evaluate the distribution of hematoporphyrin derivative (HPD) in the rat intracerebral 9L gliosarcoma brain-tumor model at 4, 24, 48, and 72 hours after intravenous administration of 10 mg/kg of the drug. Compared to surrounding normal brain, there was significant preferential uptake of HPD into the tumor. In sections surveyed, fluorescence reached a maximum value by 24 hours; however, only 33% to 44% of the tumor was fluorescent. In contrast, fluorescence within the surrounding normal brain was maximum at 4 hours, but was present in less than 1% of the brain tissue evaluated.

The effect of HPD sensitization to a laser light dose (633 nm) of 30 joules/sq cm delivered through the intact skull was evaluated histologically in 10 rats. A patchy coagulation necrosis, possibly corresponding to the distribution of HPD fluorescence seen within the tumor, was observed. There was evidence that photoradiation therapy (PRT) affects defective tumor vasculature and that a direct tumor cell toxicity spared normal brain tissue. Despite these findings, limited uptake of HPD in tumor and the brain adjacent to tumor may decrease the effectiveness of PRT in the 9L gliosarcoma brain-tumor model. Because of the similarity between the capillary system of the 9L tumor and human brain tumors, PRT may have a limited therapeutic effect in patients with malignant brain tumors.

KEY WORDS: hematoporphyrin derivative · rat brain tumor · digitized video fluorescence · photoradiation therapy

ALTHOUGH primary malignant brain tumors are usually well localized lesions that tend to recur locally, the results of treatment are generally poor. 2,17,26,32 Photoradiation therapy performed with the photosensitizing agent, hematoporphyrin derivative (HPD), and laser light have been used successfully to treat a number of non-neural malignancies. 7,8,12,16 Like other protein-bound drugs, it is thought that the photosensitizer, hematoporphyrin, and its derivatives cannot cross the intact blood-brain barrier (BBB), but can diffuse into brain tumors in which the BBB may be altered. 4,5,15,19,21,32,33 This differential accumulation of HPD in tumor tissue may allow selective sensitization of the tissue to the cytotoxic photodynamic process when tissue is exposed to the appropriate wavelength of light. 30

Experimental brain tumors have a heterogeneous population of tumor capillaries, some of which are fenestrated and highly permeable, and some of which may maintain characteristics of the normal BBB. The interface between gross tumor and surrounding normal brain may contain tumor cells that lie within the protective boundaries of an intact BBB. These regional variations in the tumor capillary bed can lead to inadequate and/or non-uniform drug delivery that can limit the intratumoral distribution of photosensitizing agents such as HPD to brain tumors; this factor may also limit the effectiveness of any form of brain-tumor chemotherapy. 20,23,24

We report here the use of a digital video fluorescence microscopy (DVFM) technique and histopathological examination to assess the uptake, distribution, and
retention of HPD in the 9L gliosarcoma rat brain-tumor model and in the brain adjacent to the tumor (BAT), a region defined by Levin, et al.20

**Materials and Methods**

**Experimental Procedure**

Left frontoparietal intracerebral brain tumors were produced in 20 male CD Fischer 344 rats, each weighing between 250 and 300 gm. A standard stereotaxic technique was used for the implantation of $4 \times 10^4$ 9L gliosarcoma cells.

The uptake and distribution of HPD was assessed in 10 rats. After the rats were anesthetized with ether, 10 mg/kg of HPD* was administered via a tail vein in two rats each at either 72, 48, 24, or 4 hours before sacrifice on Day 13 postimplantation. Two rats that were not treated with HPD (controls) were also sacrificed on Day 13 postimplantation. After sacrifice, brains were removed and 6-mm thick coronal slabs encompassing the tumor were taken. These tissue slabs, kept in the dark, were then embedded (O.C.T. compound),† and quick-frozen on dry ice. Serial coronal sections 12 µm thick were made on a cryostat at $-20^\circ$C. Adjacent alternate sections were taken, one for fluorescence studies and the other for routine histopathological examination with hematoxylin and eosin (H & E) staining.

Eight rats, six of which had been injected intravenously with HPD (10 mg/kg) 48 hours before, were anesthetized (50 mg/kg pentobarbital intraperitoneally) and exposed to laser light focused on the exposed cranium on Day 13 postimplantation. Except for the burr hole, through which tumor cells were implanted, the skull was left intact to avoid direct mechanical trauma to the tumor. The laser source was an argon pumped-dye laser‡ emitting at 630 nm. The laser energy density was 30 joules/sq cm, delivered at a power of 100 mW in a spot 2 cm in diameter over 15 minutes. Two of these rats not given HPD but exposed to laser light were the “laser-only controls,” and an additional two rats given HPD but sham-exposed to laser light served as the “HPD-only controls.” These rats were sacrificed 24 hours after laser or sham exposure. Two of the six rats treated with laser and HPD that were to be sacrificed at either 4, 12, or 24 hours after laser exposure died (see Results). After either sacrifice or death, brains were removed, inspected, and placed in 10% formalin for 1 week, after which they were fixed and embedded in paraffin. Coronal 8-µm sections were made and stained with H & E. Specimens were evaluated blindly.

**Digital Video Fluorescence Microscopy**

Fluorescent images of the coronal sections were viewed through a Zeiss RA epifluorescence/phase con-

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* HPD manufactured by Photofrin Medical Inc., P.O. Box 230, Genesee Street, Cheektowaga, New York.
† Tissue Tek II 458 embedding medium manufactured by Lab TEK Products, Naperville, Illinois.
‡ Model 770 argon laser manufactured by Cooper Lasersonics, Inc., 3420 Central Expressway, Santa Clara, California.
§ Microscope manufactured by Zeiss Instruments, 1 Zeiss Drive, Thornwood, New York.
¶ Model DAS 300 MKIII videorecorder manufactured by GYYR Products, Odetis Inc., 1335 South Clauding Street, Anaheim, California.
* Venus model TV2M video camera distributed by Zeiss Instruments, Inc., 1 Zeiss Drive, Thornwood, New York.
† Model IP5500 digital array processor manufactured by DeAnza Systems, 118 Charcot Avenue, San Jose, California.
of the image was stored in the memory of the image processor. To reduce the effect of random noise introduced by the video camera, the digital image was prepared by digitalizing and averaging eight consecutive video frames. A thresholding procedure in which an interactive “joy stick” controller was used to arbitrarily set all pixels with gray values above the threshold to a new gray value of 255 was used to identify fluorescent regions of each image for subsequent analysis. After an optimum threshold level had been determined, the same value was used for all subsequent images. The number of pixels in each total image of 396 × 512 picture elements (202,752 pixels) with gray values above the threshold was counted using the image processor. The values listed in Table 1 and plotted in Fig. 1 represent the mean and standard deviation of 20 separate measurements from each brain region (10 measurements from each brain region of two rats) for each time period studied. Locations of fluorescent areas in tissue were verified by examination of brain slices under phase contrast microscopy and by correlation with the adjacent brain section that had been stained with H & E.

Results

Fluorescence Studies

With image enhancement, fluorescence that could not be seen under routine fluorescence microscopy could be visualized. Even though DVFM has been used in subcellular drug localization studies to determine precisely the anatomic location of the fluorescence in cells, because thick cryostat-frozen sections of this highly cellular tumor were used, we did not attempt to localize fluorescence in cellular components. Results of fluorescence measurements for the four brain regions are listed in Table 1 and plotted in Fig. 1. A small amount of baseline autofluorescence was observed in the control specimens. Maximum fluorescence in normal brain could be seen along the pial-gray matter margins, choroid plexus, and the periendothelial/elastic layer within the walls of the larger blood vessels. The pattern of baseline fluorescence within the tumor was quite patchy.

In rats treated with HPD, fluorescence in the left and right hemispheres reached a maximum value by 4 hours after the HPD injection, but fluorescence within tumor and BAT did not reach maximum values until 24 hours after injection. The mean maximum fluorescence counts (that is, the area of fluorescent tissue) in the right and left hemispheres each represented slightly less than 1% of the images evaluated, whereas the maximum counts for BAT and tumor represented approximately 12% and 38%, respectively. There was a gradual decline in the fluorescence in all regions after maximum values had been reached; however, with the exception of the right hemisphere, at 72 hours after HPD injection, fluorescence in all regions was still significantly higher than levels in control rats. It is of interest that there was a slight increase in the fluorescence measured in the left hemisphere at 48 hours when compared to measurements from this region at 24 hours, which may have resulted from diffusion of HPD from the adjacent tumor region. The effect of tumor growth on fluorescence was not studied.

Within the tumors there were areas of intense fluorescence adjacent to areas of comparatively minimal or no fluorescence; however, these areas had nearly identical histological appearances on routine light microscopic examination. These differences are reflected by the magnitudes of the standard deviations for fluorescent values listed in Table 1. This appearance suggested a globular or patchy distribution of HPD (Fig. 2). Occasionally the presence of a blood vessel or sinusoidal vascular channel within or near the center of a “globule” of intense fluorescence suggested that the HPD was distributed in a perivascular pattern.

Histopathology

Control rats given either HPD plus sham-laser exposure or laser exposure without HPD were sacrificed 24 hours after surgery. These rats were alert and exhibited no change in behavior. Gross inspection of
brains revealed focal intrahemispheric tumors with an average diameter of 5 mm, which presented on the cortical surface of the left frontoparietal region. No significant displacement of the midline was observed. There was no significant necrosis, microhemorrhage, or evidence of inflammation. The lesions were typical, extremely cellular, spindle-cell malignancies with a large number of mitoses per high-power field and with finger-like invasion of the surrounding brain via the Virchow-Robin spaces (Fig. 3).

Four hours after surgery, the six rats that had been given HPD and exposed to laser were lethargic; at 12 hours the four rats that had not been sacrificed were comatose. The two rats that were to have been sacrificed at 24 hours died between 16 and 20 hours after surgery. Gross inspection of the brains from the rats sacrificed at 4 hours showed a moderate left to right shift, a feature that became more prominent in subsequent samples. Small intratumoral hemorrhages were present in the brains of one rat sacrificed at 12 hours and in one of the rats that died.

Microscopic examination of the brains from the rats treated with HPD and then irradiated had distinct focal areas of coagulation necrosis adjacent to areas of relatively usual-appearing tumor (Fig. 4). In normal brain not infiltrated with tumor, no abnormalities were identified. Less densely cellular necrotic areas that were distributed throughout the tumor were more prominent, but not necessarily confluent, near the surface where the intensity of laser light would have been greatest. These foci of necrosis were characterized by nuclear pyknosis, homogenation of chromatin, vacuolar changes within the cytoplasm, and karyorrhexis. With increasing time after light exposure there was a significant increase in the number of engorged tumor vessels. In most specimens, the vascular walls and endothelium could not be identified. Frank extravasation of red cells or vessel thrombosis was not uncommon. In the late specimens brain edema was evident at the interface between tumor and surrounding brain. Some areas seemed to have an increased number of monocytes and polymorphonucleocytes. The patchy coagulation necrosis imposed upon a background of scattered dying cells represented as much as 70% of the tumor on some sections. Frank coagulation necrosis involving the finger-like foci of tumor invading the normal brain (BAT) was not seen, although scattered dying cells were often present in these regions.

Discussion

Although not quantitative, the results obtained with DVFM indicate that compared with normal brain there was significant preferential uptake of HPD into the 9L tumor and the BAT. However, HPD was not uniformly taken up by tumor; only 33% to 44% of the tumor area was fluorescent. Maximum fluorescence was observed in normal brain and tumor tissue 4 and 24 hours after injection, respectively. Peak tumor fluorescence probably occurred between 4 and 24 hours after injection. The difference in times to peak fluorescence counts to be achieved in normal brain and tumor may have been the result of a rapid decrease in the peak plasma concentration of HPD, which could have restricted transfer of HPD across the intact BBB; however, plasma levels may have been adequate for uptake of HPD through the defective BBB of tumor regions. A prolonged or multiphasic plasma clearance of HPD, possibly related to plasma protein binding and/or enterohepatic circu-
Hematoporphyrin in rat gliosarcoma

**FIG. 4.** *Left:* Photomicrograph of a specimen of 9L gliosarcoma obtained after photoradiation therapy. Scattered dying cells are interspersed with tumor cells. H & E, × 50. *Right:* Photomicrograph showing the edge of an area of dense coagulation necrosis (upper right corner, arrows) adjacent to a comparatively normal-appearing region of tumor. H & E, × 50.

ulation, could explain the different values for \( T_{\text{max}} \). The half-life of HPD in serum has been reported to be 3 hours in mice and 24 hours in humans;\(^{13}\) because HPD is a mixture and because the degree of aggregation determines the relative presence of both lipophilic and hydrophilic components, rates of transport into and retention by different tissues of the different components may vary.\(^{18}\)

D. A. Belliner (personal communication, 1983) and others\(^{18,21}\) have found that as HPD disaggregates, an event that may take place intracellularly, both the quantum fluorescence and singlet oxygen yields are increased two- to tenfold. By using a threshold technique that was sensitive enough to detect autofluorescence in the control specimens (no HPD), the distribution of even low-level HPD fluorescence could be assessed by DVFM. Because all fluorescence above the threshold level was counted equally, an increase in the HPD fluorescence yield would not have affected DVFM counts. Only an increase in the area of tissue fluorescence — a larger area of anatomic distribution of the drug — that is the result of interstitial diffusion or increased uptake would affect the DVFM fluorescence counts. The magnitude of the increase in the fluorescence counts (from 4 to 24 hours), the consistent patchy pattern of fluorescence, and the slow loss of tissue HPD fluorescence, which is indicative of tight drug binding, suggest that an increased HPD concentration within the tumor was the source of the increase in the area of tumor exhibiting fluorescence. The finding that there is a close correlation between the concentration of HPD in tissue and fluorescence intensity supports this interpretation of our data.\(^{13,32}\) Further studies are necessary to evaluate the effect of the state of HPD aggregation within tumor on photodynamic yields.

Fluorescence in the normal brain was maximum at the pial-gray matter margin, where the cerebral blood flow is greatest and the mean intercapillary distance is least. In the 9L tumor, over 30% of the capillaries are more than 100 \( \mu m \) apart.\(^{20,21}\) The mean intercapillary distance of 80 \( \mu m \) is approximately twice that of normal rat cortex,\(^{20,21}\) and the blood flow in tumor may be reduced as much as 90% compared to normal brain.\(^{23}\) The intratumoral capillary surface area and plasma and blood volume are increased,\(^{20}\) and large sinusoidal vascular channels are frequently present. These abnormal characteristics of tissue could increase the total uptake of HPD by tumor. The relatively large intercapillary distance that increases the distance that the drug must diffuse, and the regional variations in capillary permeability could account for the patchy pattern of fluorescence observed.

Wharen, *et al.*,\(^{32}\) have suggested that 24 hours or earlier after intravenous injection of HPD may be the optimum time for exposing brain tumors to laser light. In this study, only 40% of the tumor exhibited fluorescence above the threshold level at 24 hours after injection, the period during which the maximum mean fluorescence count occurred (Table 1). Wharen, *et al.*,\(^{32}\) used tritium-labeled HPD (\(^3\)H-HPD) in their quantitative study. In contrast to their earlier findings,\(^{13}\) Gomer and co-workers\(^{14}\) have reported, and we have confirmed (unpublished results), that exchange of tritium with water and the presence of \(^3\)H-HPD contaminants restricts the sensitivity of this technique for the evaluation of the microscopic distribution of \(^3\)H-HPD in tissue. Similarly, unless high-performance liquid chromatography or another sensitive assay technique\(^{18,22}\) has been used to show that radioactive label is in the tissue-bound localizing/photosensitizing components of HPD, quantitative studies with \(^3\)H-HPD can be interpreted only with great caution. In contrast, all localizing components of HPD fluoresce and, even though they have different fluorescence yields, will not affect inter-
pretation of anatomic distribution of HPD determined by DVFM.

Significant tumor toxicity was produced by a relatively low light dose, often used clinically, delivered through the intact skull 48 hours after HPD injection. By itself, this light dose did not affect the tumor, but the presence of necrotic foci up to 5 mm deep in tumors exposed to laser light and HPD indicates that a sufficient amount of light was transmitted into tumor to produce some regional morphological effects in this model.\textsuperscript{11,24,27} The patchy pattern of necrosis seems to correlate with the pattern of fluorescence, which is consistent with the known regional variations in capillary permeability and blood flow observed in this and other brain-tumor models.\textsuperscript{15,19,34} Even though irradiating tumor through the intact skull decreased the dose of laser light delivered, this approach avoided mechanical trauma that could have affected the results. In a subcutaneous 9L gliosarcoma, Diamond, et al.,\textsuperscript{7,10} have shown that there is significant tumor enlargement in the first 30 hours after treatment with light and hematoporphyrin, presumably the result of edema, which may have been the cause of death in the rats that died in our study. The presence of edema, engorged tumor vessels, red blood cell extravasation, and/or vessel thrombosis suggests that treatment produced direct effects on the tumor vasculature. Using autoradiography, Bugelski, et al.,\textsuperscript{5} have shown that HPD became localized in the vascular stroma of tumors and that photoradiation therapy caused significant injury to the vasculature. Carpenter, et al.,\textsuperscript{9} have reported a primary, but uneven, localization of HPD around blood vessels. Using different techniques, B. W. Henderson (personal communication, 1983) and W. M. Star (personal communication, 1983) have shown that vascular toxicity is an important factor in HPD-assisted photo-radiation therapy. Broadwell and Salcman’s studies\textsuperscript{4} of the BBB in mice showed perivascular retention of a large protein marker, which can help explain the report by Rounds, et al.,\textsuperscript{26} of possible vascular toxicity in normal mouse brain from photoradiation therapy. In this study, there was no evidence of toxicity from photoradiation therapy in normal brain. The presence throughout the tumor of large areas of necrosis, scattered dying cells, karyorrhexis, and a decreased number of mitotic figures after photoradiation therapy is also consistent with direct tumor cell toxicity. A combination of “tumor bed” and direct tumor cell effects is probably active and synergistic in the in vivo mechanism that caused photoradiation therapy tumor toxicity.

Digital video fluorescence microscopy may be of value for studies of pharmacokinetics and of drug distribution that are necessary to understand the mechanisms of in vivo cytotoxicity and to plan therapeutic protocols for photoradiation therapy of central nervous system malignancies. When drug concentrations are known and the effect of HPD contaminants has been eliminated,\textsuperscript{14} DVFM can be used to obtain quantitative data. Although the technique was suggested\textsuperscript{25} and explored experimentally\textsuperscript{9,10} some years ago, photoradiation therapy is now undergoing early clinical trials for the treatment of central nervous system tumors (G. A. J. McCullough, personal communication, 1983).\textsuperscript{19,24} Unfortunately, regional variations of HPD fluorescence observed within the 9L tumor in this study suggest that inadequate drug distribution may limit the effectiveness of photoradiation therapy in the treatment of malignant brain tumors.

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References

Hematoporphyrin in rat gliosarcoma


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