Histopathological Comparison of the Effects of Hematoporphyrin Derivative on Two Different Murine Tumors Using Computer-enhanced Digital Video Fluorescence Microscopy

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ABSTRACT

A comparison study of the effects of hematoporphyrin derivative (HPD) photoradiation therapy on two different mouse sarcoma tumor model systems (RIF-1 + EMT-6) was performed. Twenty-four h after i.v. administration of HPD, the responses to total laser light doses of 50–400 J/cm² were evaluated by histological examination and the uptake and distribution of HPD using a computer enhanced digital video microscopy technique.

In response to total laser light dose (630 nm) of 50–400 J/cm², 40 mice with RIF-1 tumor showed only minimal superficial tumor necrosis upon histological examination and a 9–12% increase in maximal tissue fluorescence. In contrast, 40 mice with EMT-6 tumor showed marked areas of patchy coagulation necrosis and vascular hemorrhage at doses as low as 50 J/cm² and essentially total tumor destruction at total light doses of 150 J/cm² or more. A 59–74% increase in maximal tissue fluorescence was observed using digital video fluorescence microscopy.

It is concluded that the greater efficacy of treatment in the EMT-6 tumor as compared to the RIF-1 tumor was due to the greater localization of HPD as demonstrated by digital video fluorescence microscopy.

INTRODUCTION

There is now a considerable amount of clinical and laboratory evidence to support the conclusion that HPD³ is selectively retained by malignant tissues as opposed to the surrounding normal tissue (1–5). Since the porphyrins are highly fluorescent when exposed to UV (390–400 nm), it is possible to distinguish malignant tissue from normal tissue on the basis of fluorescence (6, 7). In addition to the ability of HPD to accumulate (and/or be retained) to a higher degree in malignant tissue and thus facilitate diagnosis, the preferential degradation of tumor tissue through photochemistry was realized. HPD has been shown to be effective in causing photodegradation of tumor tissue in experimental systems since 1972 (8) and in clinical trials since 1976 (1, 4, 5). A variety of tumors with varying histological types have been treated with HPD-PRT including cancer of the skin, esophagus, lung, bladder, eye, breast, and head and neck squamous cell carcinomas. The overall positive response rate as reported in the literature is greater than 70% (1, 4, 5).

HPD is a derivative of the heme extract hematoporphyrin HCl, prepared by successive acetylation and hydrolysis. Although the mechanism of the preferential retention of HPD is uncertain, it is known that soon after injection HPD localizes in most normal tissue, and by 48–72 h it has left the normal tissue but still remains in malignant tissue. The cytotoxicity of HPD probably results from the intracellular formation of singlet oxygen upon exposing the tumor tissue to visible light (near 630 nm). The visible radiation is generally obtained from an argon pumped dye laser although more conventional light sources have been shown to be effective (9). The dye laser output beam is coupled to quartz fibers and either applied externally to the tumor field or inserted directly into the tumor, depending on its size and site.

Many unanswered questions include delineation of light and drug dosimetry parameters, identification of the active components of HPD, mechanisms of tumor localization, and determinants of PRT cytotoxicity.

In the present work, we have carefully examined two animal tumor model systems with respect to (a) HPD retention and distribution and (b) histopathological response of the tumors to the HPD-PRT therapy. We have used computer enhanced DVFM in association with the histopathological examination in order to assess the uptake, distribution, and retention of HPD in two different mouse sarcoma tumor model systems, the RIF-1 and the EMT-6. Of particular interest was the utilization of an HPD-PRT responding mouse tumor (RIF-1) and a good HPD-PRT responding tumor (EMT-6). It was thought that this type of study would relate to human clinical studies where some tumor types responded well and others did not.

MATERIALS AND METHODS

Animal and Tumor Systems. All mice were 14–16 weeks old and weighed between 30 and 35 g at the time of treatment. The following two tumor systems were used: a RIF-1 (radiation-induced fibrosarcoma) fibrosarcoma (Department of Radiobiology Research, Stanford University, Stanford, CA) arising in the flank of a C3H/Km mouse and a EMT-6 (experimental mammary tumor) undifferentiated sarcoma (Frederick Cancer Research Institute, Frederick, MD) arising in the flank of a BALB/c mouse (10, 11). Transplanted tumors were initiated subcutaneously in the right flank of each mouse by injecting 0.1 ml of fresh tumor inoculum prepared with a concentration of 5 x 10⁵ viable tumor cells/ml suspended in RPMI (Grand Island Biological Co., Grand Island, NY). Cell viability was assessed by the ability to resist cell lysis and exclude trypan blue dye (Grand Island Biological Co.). The mouse tumors were generally palpable at 5 days and reached a size of 5–7 mm at 10–14 days at which time treatment was started.

Hematoporphyrin Derivative. HPD (Photofrin, Inc., Cheektowaga, NY) was stored in the dark and refrigerated until used. For treatment, HPD was diluted 1:4 with 0.9% NaCl solution and injected i.p.

Procedure. When tumors were of the appropriate size (as above), the animals were shaved in the tumor area and given i.p. injections of
hematoporphyrin derivative equal to 10 mg/kg body weight. All animals were kept in the normal light-dark conditions until HPD was administered. The remainder of the experiment was done in the dark, including housing of the animals. Control tumor-bearing animals included those that received no light or HPD treatment, light only, and hematoporphyrin only. Twenty-four h post-injection of HPD, the experimental animals were treated with the laser light delivery system (see below). The mouse was anesthetized with Ketamine HCl (Parke-Davis) and covered with a metal shield with a circular hole exposing the tumor. Animals were sacrificed 48 h after photoradiation therapy by halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) anesthesia. Tissue was excised immediately and fixed in 3% glutaraldehyde:5% formalin in phosphate buffer, pH 7.4. Samples were then dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Sections were cut (6 μm), stained with hematoxylin and eosin, cleared of paraffin in xylene, and dried. Sections were examined with an Axioimat microscope (Zeiss) and photographed with Panatomic-X film (Eastman Kodak).

Animals destined for fluorescence studies were sacrificed at 12 and 24 h post-injection of HPD (10 mg/kg). Tumors were excised, immediately embedded in Tissue Tek II (Miles Laboratories, Inc., Naperville, IL), and frozen at ~80°C. Sections were cut (6 μm) on a cryostat, placed on acid cleaned slides, and stored at ~80°C until fluorescence microscopy was performed.

**Laser Light Delivery System.** Laser irradiations were performed with a Coherent Innova 20 argon ion laser stimulating a Coherent PRT-95 dye laser. The dye laser was tuned to emit radiation at 630 nm. The radiation was then coupled into a 400-μm fused silica fiber optic using a Spectra-Physics Model 316 fiber optic coupler. The output end of the fiber was terminated with a microlens that focused the laser radiation into a circular field of uniform light intensity. Two types of fibers were used: one was a 600-μm fused silica fiber provided with the Spectra-Physics system; the other was a 400-μm fused silica fiber supplied by Dr. Dan Doiron. Laser irradiation emanating from the fiber was monitored with a Coherent Model 210 power meter before and after treatment.

Mice were placed underneath an aperture that controlled the area of light illumination on the tumor site. The area of illumination was approximately 1 cm². Total laser energy density ranged from 50 to 400 J/cm² with a power density of 150 mW/cm². A total of 10 control (5 HPD with no light; 5 light without HPD) and 40 light treated tumors (5 animals for each tumor model at energy densities of 50, 100, 150, 200, 250, 300, 350, and 400 J/cm²) were examined.

**Fluorescence Microscopy.** Frozen histological sections of tumor were viewed and recorded using epifluorescence. Light from a 100-W mercury lamp was filtered through a G-546 band pass exciter filter (Zeiss) and directed to the tissue by a chromatic beam splitter. The fluorescence was filtered through a LP-590 long pass barrier filter and then directed to a low light level video camera, Venus Scientific No. TV2M (Zeiss). The video signal was acquired on video tape by a GYYR DAS-MkII video tape recorder.

**Image Processing.** The image processing system used to analyze the fluorescence recorded above has been described earlier (12). The signal from the video tape recorder in playback mode was fed into an image array processor with synchronization between the two units provided by a time base corrector. A LSI-11/23 minicomputer was used to control the image processor.

The video signal was acquired by the image processor under control of software loaded in the computer. Thirty-two frames were acquired from each video scene and then averaged to give an increase in the signal:noise ratio. This acquired image was used by the computer for measurement of the average gray level (corresponding to fluorescence) within a zone defined by a cursor on the image display monitor. Measurements were made in all areas of the tumor tissue. Generally the tumor was arbitrarily divided into four equal areas, and within each area 10 measurements were made. The average of the 40 measurements is presented in the tables. Ten animals in each group were examined. In all cases, the experimental fluorescence values were compared to fluorescence values from similar tumors in animals not receiving HPD injection.

**RESULTS**

**Histopathology**

**EMT-6.** In contrast to the RIF-1 tumor model, those animals that received total doses of light equal to 50 J/cm² showed frank evidence of gross hemorrhage throughout the tumor tissue and almost total destruction of the tumor tissue was seen at total light doses of 150 J/cm² or more. Histologically at total light doses of 50 J/cm² the tumor vessels were engorged with vascular thrombosis and exhibited frank extravasation of RBC into the surrounding tissue stroma (Fig. 1, a and b). At doses of 150 J/cm² or more, there was 100% destruction of the tumor architecture characterized by nuclear pyknosis and karyorrhexis, with only minimal preservation of the basic cellular shape permitting recognition of the cellular outline in a sea of amorphous granular debris (see Fig. 1, c and d).

**RIF-1 Tumor.** Gross inspection of the tumors revealed no visual evidence of necrosis in the controls and only minimal superficial necrosis at the surface of the tumor in those animals that received both HPD and light. There was little difference in the percentage of necrosis in those animals that received a total light dose of either 50 or 400 J/cm². Histological examination of tumors from mice receiving HPD and light showed only small focal areas of coagulation necrosis adjacent to the surface of the tumor (Fig. 2). In the deeper areas of the tumor, no abnormalities were identified and the normal tumor architecture was preserved (Fig. 2, b and d).

**Computer Enhanced Digital Video Fluorescence Microscopy**

**EMT-6.** Animals were sacrificed at 12 and 24 h post-injection of HPD (10 mg/kg) and the total fluorescence was again measured by DVFM. Relative to tumor bearing non-HPD control animals, the increases in total tumor tissue fluorescence were 59–62 and 66–74% at 12 and 24 h, respectively. Within these tumors, there was a diffuse increase in fluorescence throughout the tumor stroma with additionally intense areas of fluorescence associated in regions of the microvasculature (Table 1).

**RIF-1.** Animals receiving HPD (10 mg/kg i.v.) were sacrificed at 12 and 24 h postinjection and the relative fluorescence of the tumor was measured by DVFM. These tumors showed a relative 9–10 and 11–12% increase in total fluorescence as measured against tumor bearing control animals that did not receive HPD (Table 2).

**DISCUSSION**

Hematoporphyrin derivative has shown great promise in the selective photodegradation of malignant tissue while sparing adjacent normal tissues. It has been found to be effective, within limits, in the treatment of a large number of carcinomas in both human patients and animal models. This is an area of intense research interest which is currently being investigated by numerous groups throughout the world.

It is evident that HPD is being picked up by both normal and malignant tissues by 12 h and probably occurs maximally around 24 h. In cell cultures, the total amount of HPD taken up does
HISTOFLUORESCENCE OF HPD IN TUMORS

Table 1
Results of DVFM measurements in EMT-6 tumor

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<th>Mean absolute fluorescence (pixels)</th>
<th>Av.</th>
<th>Increase from control</th>
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<tr>
<td>Trial 1 Control</td>
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<td>24 h</td>
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<td>71.83</td>
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% of increase from control

Table 2
Results of DVFM measurements in RIF-1 tumor

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<th>Mean absolute fluorescence (pixels)</th>
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<th>Increase from control</th>
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% of increase from control
vary from cell line to cell line. In addition, it cannot be simply stated that relatively more HPD is being accumulated by malignant tissue. It appears that the total time that HPD is being retained in the malignant tissue is much longer than in nonmalignant tissue. As a result, there is a "window" of time in which one can exploit the differences in HPD concentration to bring about the selective photodegradation of the malignant tissue. The binding sites of HPD (or its active ingredient) have been postulated by various authors to be the cell membrane (13), mitochondria (14), and lysosomes (15). However, a clear understanding of the exact cellular site of the photochemistry is not available at the present time.

A great deal of recent work has implicated the tumor vasculature as a major site of tumor destruction. Bugelski et al. (16) demonstrated autoradiographically using tritium-labeled HPD that the concentration of HPD in the perivascular stroma is 5 times that in other tumor cells. Carpenter et al. (17) also reported primary localization of HPD around blood vessels. These data all implied that the endothelial cells surrounding the blood vessels played a central role in determining the extent of tissue damage caused by HPD-PRT. In contrast to these studies, tissue culture results (14) and DVFM of rat brain tumors (18) implicate direct HPD binding to tumor cells in the phototoxic response.

Our histological data support both vascular photodestruction and direct tumor cell killing. Furthermore, the histopathological analysis was directly correlated with tumor fluorescence as determined by computer enhanced digital video fluorescence microscopy. The results with DVFM indicate that, compared to control non-HPD treated tumor tissue, there was a significant increase in the uptake, localization, and retention of HPD in the EMT-6 model as opposed to the RIF-1 model (70% as compared to 10% measured at 24 h). The magnitude of this increase in tissue fluorescence compared to controls suggests that an increased concentration of HPD within the tumor tissue was responsible for the relative increase in total tissue fluorescence. Furthermore the close correlation between increased tissue fluorescence and increased tissue destruction as observed histopathologically supports this conclusion. Fluorescence within the tumor tissue was maximal at the tumor surrounding tissue interface and along the tumor microvasculature suggesting that this is where intratumoral blood flow is the greatest. The earliest evidence of significant tumor toxicity produced by relatively low light doses was marked thrombosis and hemorrhage of the tumor microvasculature. This seems to correlate with the observed pattern of fluorescence. Furthermore it is very possible that a differential amount of tumor vasculature in the two models studied could explain the results.

The results of this study emphasize the importance of tumor vasculature in the success of HPD-PRT and further suggest that tumor destruction is due to both destruction of the vasculature and a phototoxic response in individual dividing tumor cells. In addition, this study demonstrates a clear correlation between tumor fluorescence and histopathological photodegradation. It is clear that the RIF tumor, which is relatively resistant to HPD-PRT, does not readily incorporate HPD as indicated by low levels of fluorescence. This result contrasts sharply with a paper published subsequent to the submission of our study (19). In that study, no difference in HPD content was detected between either the RIF-1 or the EMT-6 tumor using fluorescence measurements on gross tumor slices or tissue absorbance measurements.
However, similar to our study, the EMT-6 tumor was much more sensitive to HPD-PDT. Since no histological micrographs were presented in that study, it is impossible to validate the observations. Furthermore the method used to detect fluorescence was not on frozen histological sections but on the gross cut tumor surface. This method was far less sensitive than the DVFM technique used in our study, which involved making 40 individual measurements in 5 different tumors for each group studied. Furthermore the low light level tissue excitation used to stimulate fluorescence in our study was far less likely to cause artifacts due to fluorescence bleaching by the excitation wavelengths. In addition, the sensitive SIT camera used in our study would be far more likely to detect fluorescence intensity above the background tumor fluorescence than the method reported in the study of Henderson et al. (19). This is an important point since it is well known that tumors concentrate natural porphyrins thus resulting in a high inherent level of porphyrin fluorescence.

REFERENCES

Fig. 1 Photomicrographs of EMT-6 tumor removed 48 h after treatment with HPD and light at total doses of 50 J/cm² (a and b), 150 J/cm² (c and d). Note evidence of extravasation of RBC into surrounding perivascular tumor stroma at the tips of the arrows at a dose of 50 J/cm² (a and b) and almost total destruction of the tumor tissue at a dose of 150 J/cm² (c and d). a and c, × 200; b and d, × 500.
Fig. 2. Photomicrographs of RIF-tumor removed 48 h after treatment with HPD and light at total doses of 50 J/cm² (a and b) and 150 J/cm² (c and d). Note only minimal superficial coagulation necrosis at the tip of the arrow adjacent to the surface of the tumor only in c at a total light dose of 150 J/cm². Note normal tumor architecture with mitotic figures present at the tips of the arrows at both doses, 50 and 150 J/cm², (b and d). a and c, × 200; b and d, × 500.
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