Title
Photodynamic therapy of hypervascular cutaneous tissues in animal models using porphyrin or phthalocyanine activated by red light

Permalink
https://escholarship.org/uc/item/3504f8bw

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Publication Date
1990-06-01

DOI
10.1117/12.17453

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Peer reviewed
ABSTRACT

Continued improvement in the results of laser treatment of port-wine stain (PWS), with reduction in scarring, will depend on the ability to use lasers to induce selective injury of only the abnormal blood vessels in the dermis. Photodynamic therapy (PDT), using an exogenous photosensitizing drug specifically activated by a certain wavelength of light, may be used to destroy selectively certain targets in biological systems. The current study demonstrates, in the chicken comb animal model, that PDT using porphyrins and phthalocyanines activated by red light could be used to treat hypervascular dermal tissues.

2. INTRODUCTION

Cutaneous vascular anomalies such as port-wine stains (PWS) have been treated in the past with a large array of therapeutic modalities including scalpel surgery, ionizing radiation, skin grafting, dermabrasion, cryosurgery, tattooing, and electrotherapy. All of the above have met with but limited success and have often left cosmetically unacceptable scarring.1

More recently, the laser has offered a superior approach in therapy due to its ability to destroy selectively cutaneous blood vessels. Blue-green or yellow light produced by the laser will pass through the epidermis and then be preferentially absorbed by oxyhemoglobin (the major chromophore in blood vessels) in the ectatic venules in the upper dermis. There, the photon energy will be converted to heat causing thermal damage and thrombosis in the targeted vessels. The degree of selectivity depends on the various laser parameters such as wavelength, pulse duration and energy density which are inherent in each type of laser.2,3

Presently all laser systems used in the treatment of hypervascular cutaneous anomalies effect tissue change through a photothermal mechanism. As a result, current devices have the potential to produce undesirable skin surface textural changes including hypertrophic scarring, atrophy, induration, and hypo- or hyperpigmentation if this heating process is not precisely controlled. Due to these inherent risks, it is obvious that a
method which allows the precise destruction of only blood vessels without the production of heat would have a distinct advantage over existing techniques.

The current study examines the feasibility of photodynamic therapy (PDT), using the photosensitizers Photofrin II (HpD) or chloroaluminum sulfonated phthalocyanine (CASpc), specifically activated by red light at the appropriate therapeutic wavelength and intensity, to destroy selectively cutaneous blood vessels in the chicken comb. Light provides the energy to drive a series of photochemical reactions, without the generation of heat, and to destroy cutaneous blood vessels. The comb was chosen as the model because its histoanatomy is analogous to that found in hypervascular cutaneous anomalies (including PWS). This approach certainly has a successful parallel in animal and human oncology where PDT has been shown to cause destruction of the tumor microvasculature.

3. MATERIALS AND METHODS

3.1 Animal Model

Adult, female Leghorn chickens weighing between 2.5-3.5 kg at the time of treatment were used. After injection of the photosensitizer, the remainder of the experiment, including housing of the animals, was conducted in the dark.

3.2 Photosensitizers

Photofrin II (HpD) was obtained from Photomedica, Inc. (Raritan, N.J.) as an aqueous solution at a concentration of 2.5 mg/ml and stored in the dark at -70°C until used. Prior to injection, HpD was quick thawed and allowed to come to room temperature for 1 hour. For in vivo experiments, animals received single intravenous injections in doses equal to 10 mg/kg body weight.

Chloroaluminum sulfonated phthalocyanine (CASpc) was obtained from the Nuclear Research Center (Negev, Israel), as a dark blue powder. Prior to injection, CASpc was quick thawed, reconstituted in PBS, and allowed to come to room temperature for 1 hour. For in vivo experiments, animals received single intravenous injections in doses equal to 1 mg/kg body weight.

These drug doses were predetermined and based on work previously completed to examine the effects of PDT on malignant tumor microvasculature.4

3.3 Laser Li•ht Delivery System

Laser irradiations were performed with a Cooper Lasersonics
(Santa Clara, CA) 770 DL argon pumped dye laser. The dye used was DCM Pre-Mixed Laser Dye (Cooper Lasersonics) with a tuning range of 610-690 nm. The dye laser was tuned to emit radiation at 630 nm for use with HpD and 675 nm for CASPc. The wavelengths were verified to ± 1 nm using a Clinical Hartridge Reversion Spectroscope (Ealing Electro-Optics, South Natick, MA). The radiation was then coupled into a 400 μm fused, silica fiber optic using a Spectra-Physics (Mountain View, CA) Model 316 fiber optic coupler. The output end of the fiber terminated with a microlens that focused the laser radiation into a circular spot of uniform light intensity. Laser irradiation emanating from the fiber was monitored with a Coherent (Palo Alto, CA) Model 210 power meter before and after treatment.

3.4 Experimental Procedure

The first part of the study was to determine the optimal time interval for laser irradiation after injection of the photosensitizer. It was assumed that if tissue was irradiated immediately after intravenous administration of the photosensitizer, the drug would have distributed no further than the vascular compartment, thus potentiating the selective destruction of blood vessels. Animals were given intravenous injections of HpD or CASPc and then acutely, 1 hr., 2 hrs., 3 hrs. or 4 hrs. later, a 1 cm² area of the comb was irradiated. Total light dose was 20 J with a power density of 90 mw/cm². For the determination of the optimal post-injection time interval a low total light dose was chosen deliberately in order to be close to the threshold for blanching. Blanching of the irradiated area was documented by daily photography for 2 weeks. The HpD treated animals that received light 2 hrs. post injection showed the most prolonged blanching of their combs while the CASPc treated animals showed the most prolonged blanching at 1 hr. post injection irradiation (see results). Thus these time intervals were fixed for the remainder of the experiment. No histologic studies were conducted on these combs.

The objective of the second part of the study was to determine the relevant light parameters necessary to produce long-term blanching of the comb. Animals were given HpD or CASPc, and 2 or 1 hrs. later respectively, two 1 cm² areas of the comb in each animal were irradiated with total light dose of 50 J with a power density of 90 mw/cm². Control groups received light without photosensitizer, or HpD or CASPc alone without light; in the same dosages and wavelengths as the groups with HpD or CASPc and light. Blanching of the irradiated area was documented by daily photography.

Additionally, further groups of animals received photosensitizer in dosage of 10 mg/kg HpD or 1 mg/kg CASPc and exposed to total light dose of 50 J with a power density of 90 mw/cm². Biopsies were taken from combs in animals sacrificed by intravenous injection of Euthasix (Western Medical Supply, Arcadia, CA) acutely, 24 hrs., 3
days, 7 days, 14 days and 28 days after light treatment. Biopsy tissue material was fixed in Karnovsky's fixative (2% paraformaldehyde: 3% glutaraldehyde) and subsequently embedded in propyleneoxide-Epon 812 substitute [Poly/Bed 812 Embedding Media (Polysciences Inc., Warrington, PA)]. Five hundred nanometer sections were cut and stained with Richardson's stain. Sections were examined with an Olympus microscope and photographed with Panatomic-X film (Eastman Kodak Co., Rochester, NY).

4. RESULTS

4.1 Gross Observations

Control combs showed no blanching at any of the photosensitizer and light parameters used. In the PDT group examined acutely, the irradiated area appeared darkened. At 24 hrs (Fig. 1), the irradiated area showed blanching which persisted for 21-28 days. After this time interval, the irradiated area revascularized centripetally until the treated area had resumed its normal color. These observations were consistent for both the HpD and CASPc treated combs.

4.2 Light Microscopy

Control slides (Fig. 2) showed the normal comb architecture with large multiple venules, having different lumen diameters sparsely filled with nucleated discoid erythrocytes, in the upper dermis. The endothelial cells lining the venules appeared elongated and flat. In the PDT group examined acutely (Fig. 3), the most striking change, compared to controls, noted at this point was occlusion of the venule lumen with tightly packed erythrocytes compressing the endothelial cells laterally. At 24 hrs. post PDT (Fig. 4), the vascular lumen was occluded with debris, "ghosts" from deteriorating erythrocytes, and organizing thrombus. The endothelial cells of the venules were markedly swollen. In these cells, the nucleus was lighter stained and the heterochromatin dispersed. The cytoplasm contained numerous large vacuoles indicative of impending cell death. At 3 days post PDT (Fig. 5), the lumen remained occluded with deteriorating cellular fragments and organizing thrombus. The endothelial cells were absent. At 7 and 14 days post phototherapy (Fig. 6), the vascular lumen was distinguishable but vacant of any blood elements. No endothelial cells were visible. After these intervals, gradual revascularization was seen and essentially completed by 28 days. These observations were consistent for both the HpD and CASPc treated combs.
The basic concept of PDT is that certain molecules can function as photosensitizers. The presence of the photosensitizer in certain targets makes the latter vulnerable to light at a wavelength absorbed by the photosensitizer. The action of photosensitizers is to absorb photons of the appropriate wavelength sufficient to elevate the sensitizer to an excited state. The excited photosensitizer subsequently reacts (exchanges its energy) with a molecular substrate, such as oxygen, to produce highly reactive singlet oxygen which causes irreversible oxidation of some essential component. All of these photochemical reactions occur without the production of heat.

During the past several years, many photosensitizing porphyrins have been used to treat selectively solid tumors in humans and mammals. It is estimated that worldwide more than 3000 patients with a variety of malignant tumors have been treated with PDT. Histology of PDT treated tumors shows that apparent internal hemorrhage and red cell extravasation are common findings after PDT, not only in most experimental animal tumors but in tumors in patients as well. This observation suggests that the effects of PDT leading to rapid necrosis of tumor tissue are not the result of direct tumor cell kill but are secondary to destruction of the tumor microvasculature. Although photodynamic effects on the microvasculature of tumors have been well documented, the idea of exploiting PDT for the therapy of cutaneous vascular anomalies is novel. The objective of this study was to develop a protocol for the localization of PDT effects in hypervascular cutaneous tissues using the photosensitizers HpD and CASPc.

The first question that needed to be addressed was - when is the photosensitizer maximally concentrated in the target vasculature? It was hypothesized that if tissue was immediately irradiated after intravenous administration of photosensitizer, the drug would have distributed no further than the vascular compartment, thus improving on the partial selectivity associated with conventional photothermal therapy. In the initial set of experiments the time interval between photosensitizer injection and irradiation ranged from acute to 4 hrs. It was determined that a 2 hr. time interval for HpD and 1 hr. interval for CASPc caused the most pronounced and persistent blanching. This interval represents the time necessary for a requisite concentration of photosensitizer to be distributed adequately in the targeted vasculature.

With the 2 hr. maximum effect intervals defined, the effects of PDT were examined grossly and histologically. Control animals (light without photosensitizer) showed no blanching response in the comb. This eliminated any possibility that photothermal effects occurred at the irradiation parameters used. Consequently, the effects observed in this study must be of a photochemical nature.
All treated combs (light with photosensitizer) demonstrated a darkening phase immediately after phototherapy due to occlusion of the venule lumen by tightly packed erythrocytes. Within 24 hrs. after light treatment, the comb blanched. Histologically, this correlated with the venule lumen being occluded with debris and "ghosts" of deteriorating erythrocytes and organizing thrombus. Early signs of cell death were also noted in the endothelial cells lining the venule lumen. From 24 hrs., through 21-28 days, the comb remained blanched. After these intervals, revascularization of the comb was seen, grossly and histologically, and essentially completed by 28 days. These observations were consistent for both the HpD and CASPc treated combs.

With the present protocol, an effective, albeit temporary, blanching of the hypervascular comb was achieved. Longer term blanching may be achieved once light and photosensitizer dosimetry parameters have been completely defined. However, it may prove impossible to achieve permanent blanching of the comb due to the inherent natural tendency of the injured comb to revascularize. The comb, being important in controlling the animal's natural body temperature, possesses a large high pressure vascular network which may be sufficient to revascularize any occlusion produced by PDT. This may not be a consideration in the human patient where the PWS vessels represent a very low pressure system which could be effectively occluded by PDT.

In conclusion, this study demonstrates that the photosensitizers HpD and CASPc activated by red light at the appropriated therapeutic wavelengths, can cause selective damage and occlusion of vessels in the hypervascular comb. This occurs by a photochemical rather than a photothermal mechanism. Based on the results of this study it may be possible to use this approach in the treatment of cutaneous vascular anomalies such as PWS in humans and a clinical trial may be merited.

6. ACKNOWLEDGMENTS

The authors wish to thank Giselle P. Lim for her superb preparation of the histology slides and photographs and to Dr. Ehud Ben-Hur for supplying the CASPc. This study was supported by NIH grants 5P41 RR0192-09 and 5 RO1 CA 32248-06, and Department of Defense grant SDIO84-88-C-0025.

7. REFERENCES


8. FIGURES

Fig. 1: Chicken comb injected with HpD 24 hrs. after irradiation with 630 nm light; X 3.
Fig. 2: Histologic section of control (no photosensitizer, no light) comb; X 390.

Fig. 3: Histologic section of comb injected with HpD examined acutely after irradiation with 630 nm light. Note occlusion of the venule lumen with tightly packed erythrocytes; X 390.
Fig. 4: Histologic section of comb injected with CASPc examined 24 hrs. after irradiation with 675 nm light. Note occlusion of the venule lumen with debris, "ghosts" of deteriorating erythrocytes, and organizing thrombus. The endothelial cells are markedly swollen; X 390.

Fig. 5. Histologic section of comb injected with CASPc examined 3 days after irradiation with 675 nm light. Note persistent occlusion of the venule lumen with deteriorating cellular fragments and organizing thrombus. The endothelial cells were absent; X 390.
Fig 6. Histologic section of comb injected with HpD examined 2 wks. after irradiation with 630 nm light. Note large vascular lumen vacant of any blood elements. No endothelial cells are visible. X 390.