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Photodynamic Therapy of Human Malignant Melanoma Xenografts in Athymic Nude Mice[1,2]

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While photodynamic therapy (PDT) for cutaneous malignancies including dermal recurrences of breast cancer and basal cell carcinomas has shown great promise, PDT of malignant melanoma has remained incompletely understood. A comparison study of the effects of PDT on human xenograft amelanotic and melanotic malignant melanoma in the athymic nude mouse model was performed. Twenty-four hours after ip administration of Photofrin II, the responses to total laser light doses of 25-300 J/cm^2 were evaluated by histologic examination. Animals were also sacrificed 24 hours after administration of Photofrin II without light, and their uptake and localization of hematoporphyrin derivative (HpD) for each tumor were measured and compared. The results indicate that human xenograft melanotic melanoma, despite the fact that it contains more HpD than does amelanotic melanoma, is far less responsive to PDT. This result appears to be due to the competing chromophore melanin, which may inhibit the photochemical reaction at several key points. [J Natl Cancer Inst 1988;80:56-60]

The basic concept for the use of PDT in the treatment of malignant tumors is that certain molecules can function as photosensitizers. The presence of these photosensitizers in certain cells thus makes these cells vulnerable to light at the appropriate wavelength and intensity. The action of these photosensitizers is generally to absorb photons of the appropriate wavelength sufficient to elevate the sensitizer to an excited state. The excited photosensitizer subsequently reacts with cellular oxygen and results in the production of active cytotoxic radicals leading to tumor destruction. While numerous compounds have been tested as selective photosensitizers of malignant cells, HpD has received the most attention as a selective-tumor-localizing photosensitizer (*1*).

While PDT can be used to reduce relatively large tumors, it appears to be especially advantageous in the treatment of thin superficial tumors easily accessible to light. Since photosensitizers have been used in dermatology to enhance the therapeutic effects of light in treating a wide variety of disorders including vitiligo and psoriasis, a logical extension of PDT would seem to be in the local treatment of malignant lesions involving the skin. While good results have been reported in the treatment of dermal recurrences of breast cancer and basal cell carcinomas (*2*), PDT of malignant melanoma has not been demonstrated.

The purpose of this study is to compare the effectiveness of PDT in causing selective tumor necrosis of human xenograft amelanotic and melanotic malignant melanoma in the athymic nude mouse model. Several reports have indicated that the histology, growth characteristics, biologic behavior, and response to chemotherapeutic agents and other treatment modalities of melanoma xenografts are maintained after serial transplantation in athymic nude mice (*3-5*). It is hoped that this type of study would relate to human clinical studies where some melanomas responded well and others did not.

Materials and Methods

Hematoporphyrin derivative. Photofrin II was obtained from Photomedica Inc., Raritan, NJ, as an aqueous solution at a concentration of 2.5 mg/ml and stored in the dark at −80°C until used. For in vivo experiments, Photofrin II was diluted 1:4 with 0.9% NaCl solution and injected ip.

Animal and tumor system. The congenitally athymic (*nu/nu*) mouse experimental model for human xenograft melanoma has been well described (*6*). Metastatic human amelanotic and melanotic melanoma was harvested fresh from surgical specimens. It was maintained in vivo by serial transplantation in athymic nude mice. For this experiment, tumors were harvested fresh from mice and minced with fine scissors. Transplanted tumors were initiated id in the flanks of each mouse by injecting 0.1 ml of small tumor fragments suspended in RPMI (Grand Island Biological Co., Grand Island, NY). Mouse tumors were allowed to reach a size of 5-7 mm, at which time treatment was started. At this size, the small tumor was homogeneously white and spontaneous tumor necrosis minimal or absent.

Procedure for photosensitization studies. When tumors were of the appropriate size (as indicated above), the animals were shaved in the tumor area and given ip injections of Photofrin II in doses equal to 10 mg/kg (body wt). The remainder of the experiment was done in the dark, including housing of the animals. Control tumor-bearing animals were those that received light without sensitizer and sensitizer without light. Twenty-four hours post injection of Photofrin II, the experimental animals were treated with the laser light delivery system (*see* below). The mouse was anesthetized with Ketamine HCl (Parke, Davis & Co., Detroit, MI) and covered with a metal shield with a circular hole exposing the tumor. Animals were sacrificed 24 hours after PDT

ABBREVIATIONS USED: HpD = hematoporphyrin derivative; PDT = photodynamic therapy.

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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. Six-micrometer sections were cut, stained with hematoxylin and eosin, cleared of paraffin in xylene, and dried. Sections were examined with an Axiomat microscope (Carl Zeiss, Inc., New York, NY) and photographed with Panatomic X film (Eastman Kodak Co., Rochester, NY).

Laser light delivery system. Laser irradiations were performed with a 770 DL argon dye laser system (Cooper Lasersonics, Santa Clara, CA). The dye used in the dye laser was DCM premixed laser dye (Cooper Lasersonics) with a tuning range of 610-690 nm. The dye laser was tuned to emit radiation at 630 ± 1 nm for the entire experiment. The wavelength was verified using a #5/354 UV monochromator (Jobin Yvon, Longjuneau, France). The radiation was then coupled into a 400-μm fused silica fiber optic by using a model 316 fiber optic coupler (Spectra-Physics, Inc., Mountain View, CA). The output end of the fiber was terminated with a microlens that focused the laser irradiation into a circular field of uniform light intensity. Laser irradiation emanating from the fiber was monitored with a Coherent model 210 power meter before, during, 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 1 cm^2 with a power density of 100 mW/cm^2.

Animals with either the human xenograft amelanotic or melanotic melanoma were treated with the following doses of light: 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, and 300 J/cm^2. Animals were sacrificed 24 hours after treatment, and their tumors removed for histopathologic analysis as described above.

Localization and uptake studies. Five animals with tumors of either amelanotic or melanotic melanoma, which were destined for localization and uptake studies, were sacrificed 24 hours post injection of doses of Photofrin II at 10 mg/kg. Tumors were excised and immediately frozen at −80°C until extraction procedures were performed. The extraction procedure used has been previously described by Kessel (*7*). Tumor tissue was quick thawed and weighed (≈300-600 mg wet wt). Extractions were carried out by disrupting tumor tissue in 2.5 ml sodium phosphate buffer (pH 3.5) with the use of a glass homogenizer. The homogenate was shaken for 5 minutes at 22°C with 2.5 volumes of 1:1 methanol-chloroform and subsequently centrifuged (1,000*g*, 10 min, room temperature). The lower fluorescent phase was removed and evaporated under nitrogen, the residue was taken up in 100 μl of methanol, and insoluble materials were removed by brief centrifugation (12,000*g*, 30 sec, room temperature). Porphyrin uptake was estimated from the absorbance of a 2-ml aliquot of the methanol extract scanned from 350 to 650 nm with the use of a Beckman DU-7 spectrophotometer. The porphyrin concentration of each tumor was determined by comparing its absorbance at 400 nm with a known concentration of Photofrin II (*8*). Absorption spectra were obtained in solution for each tumor, which showed a broad peak of maximal absorption between 380 and 420 nm. Values listed in table 1 are expressed in terms of microgram porphyrin per gram tumor tissue (wet wt).

Results

Histopathology. (*a*) Amelanotic melanoma: Gross and microscopic examination of tumors revealed no evidence of necrosis in control animals (light without sensitizer and sensitizer without light). Histologically, at total light doses of 75 J/cm^2 or more, there was 100% destruction of the tumor 24 hours after PDT (fig. 1). The tumor cells that were not completely disrupted showed nuclear pyknosis and karyorrhexis with only minimal preservation of the basic cellular outline in a sea of red blood cells and amorphous granular debris.

(*b*) Melanotic melanoma: In contrast to the amelanotic tumor, melanotic melanoma that received total doses of light equal to 75 J/cm^2 showed only superficial necrosis with hemorrhage to a depth of 2-3 mm below the surface of the tumor (≈30%-40% of the total tumor vol) (fig. 2). Deep to this damaged zone was normal-appearing tumor with intact vasculature. Tumors that received up to 150 J/cm^2 of light demonstrated necrosis to a depth of 4-5 mm (60%-80% of the tumor vol) with resistant tumor present at the base and periphery of the tumor. However, within this necrotic zone, isolated patches of normal-appearing tumor cells were observed to be present, indicating PDT-resistant tumor (fig. 3). Tumors that received 200-300 J/cm^2 showed complete necrosis to the base of the tumor centrally. At the anteroposterior and lateral margins of the tumor, histology confirmed viable nests of persistent tumor cells (fig. 4). This conclusion is based upon the histopathologic examination of a large number of sections and tumors and is not attributable to the way a particular section was cut.

Table 1. HpD tumor localization and uptake in human xenograft amelanotic and melanotic melanoma

Tumor	Uptake μg porphyrin/g tumor	Average μg porphyrin/g tumor	Increase from control
Control (non-HpD)			
Amelanotic	1.03	1.04	1.0
	1.05		
Melanotic	1.07	1.09	1.0
	1.11		
Amelanotic	7.28	7.44	7.15×
	7.67		
	7.58		
	7.36		
	7.31		
Melanotic	8.26	8.42	7.72×
	8.61		
	8.41		
	8.28		
	8.55		

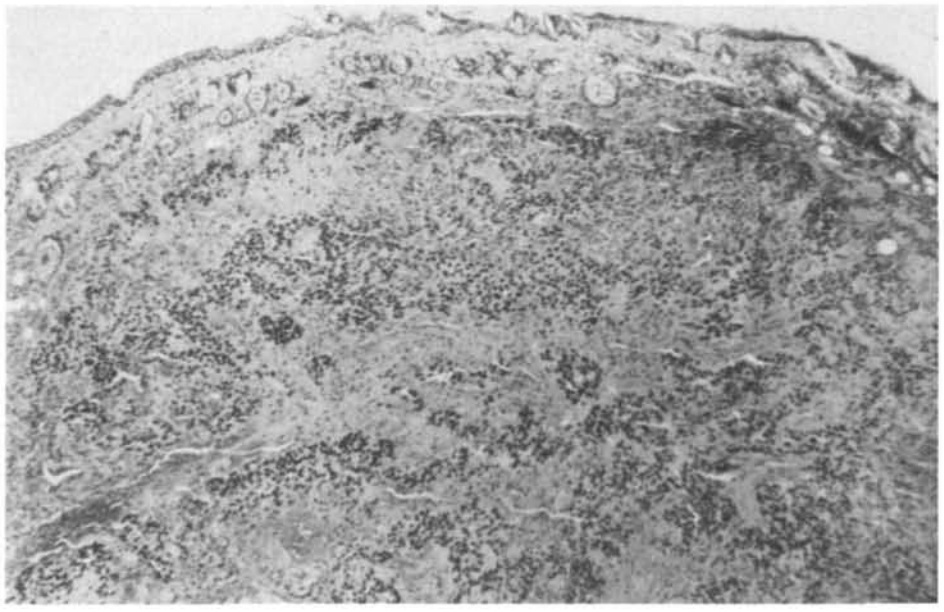

Figure 1. Photomicrograph of human xenograft amelanotic melanoma tumor removed 24 hr after treatment with Photofrin II and 75 J/cm^2 of light. At this dose of light the entire tumor is destroyed by hemorrhagic necrosis. Originally, ×50.

Tumor localization and uptake. Animals were sacrificed and their tumors removed 24 hours post injection of 10 mg/kg of Photofrin II. The porphyrin was extracted and expressed in terms of microgram porphyrin per gram tumor (wet wt), and tumors were compared in terms of their increase over control non-HpD tumors. Control amelanotic tumors contained an average of 1.04 μg porphyrin/g tumor. Amelanotic tumors that received HpD had an average of 7.44 μg porphyrin or 7.15 times that of control tumors. Melanotic control tumors contained 1.09 μg porphyrin, while melanotic tumors that received HpD had an average of 8.42 μg porphyrin or 7.72 times that of control tumors (table 1).

Discussion

The usual treatment of primary malignant melanoma is surgical, and the first neoplasm treated experimentally with a laser "scalpel" was a malignant melanoma (*9*). Early laser experiments on malignant melanomas consisted of photoexcision and photocoagulation of both primary and metastatic lesions. The necrosis was eventually replaced by scars felt to be more cosmetically acceptable than those following scalpel or electrosurgery. However, the long-term findings from these studies were largely negative and unproductive (*10*). Within the next few months, numerous new nodules appeared along the borders of excision due to incomplete resection. PDT, since it may provide a greater degree of selectivity than other treatment modalities currently being applied to these cases, offered new hope in targeting those viable tumor cells that may tend to remain behind after conventional treatment.

An important parameter determining the extent of tumor necrosis produced by PDT is the penetrability of the necessary visible light through the tissue being irradiated. This penetration will be dependent on the reflection, scattering, transmission, and absorptive characteristics of the particular tissue being exposed. Since PDT is a photochemical reaction rather than a thermal ablative or coagulative process, the absorptive properties of the tissue that depend on the specific chromophores present in the absorbing tissue will obviously be important. The energy of the photon is absorbed by the chromophore, which can then transfer its energy by various mechanisms to the target molecule. In the case of PDT, tumor necrosis is thought to be produced by the energy transfer from the excited triplet state of the porphyrin to oxygen, producing singlet oxygen, which causes irreversible oxidation of some essential cellular component (*11*). If, however, another chromophore is present in the tissue that has a strong absorption band located at the particular wavelength used, this chromophore will compete with the photosensitizer for photons resulting in inefficient phototoxicity.

Melanin is a stable protein-polymer complex with a broad absorption spectrum over 250–1,200 nm (*12*). It is synthesized into the skin by melanocytes and is sometimes referred to as a "light

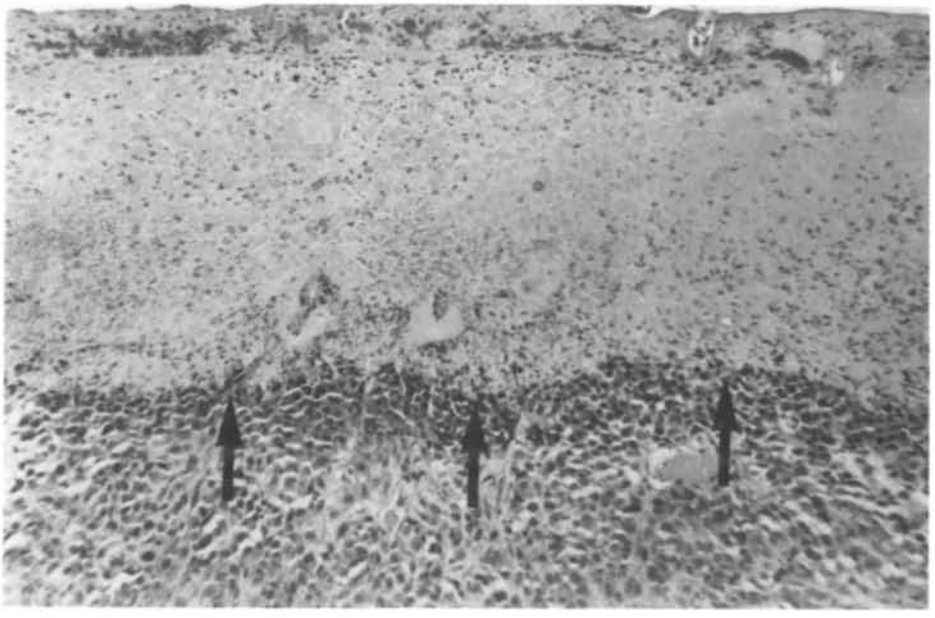

Figure 2. Photomicrograph of human xenograft melanotic melanoma tumor removed 24 hr after treatment with Photofrin II and 75 J/cm^2 of light. Note evidence of superficial hemorrhagic necrosis above the arrows. Deep to this damaged zone is normal-appearing tumor. Originally, ×80.

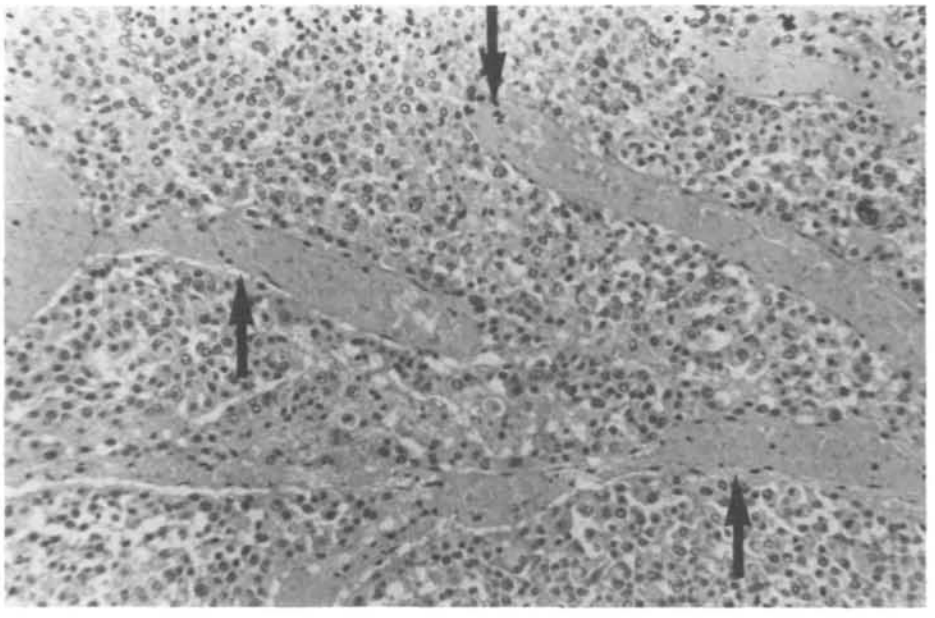

Figure 3. Photomicrograph of human xenograft melanotic melanoma tumor removed 24 hr after treatment with Photofrin II and 150 J/cm^2 of light. Note the large areas of hemorrhagic necrosis throughout the tumor at the tips of the arrows. However, within this necrotic zone isolated patches of normal-appearing tumor cells are present indicating PDT-resistant tumor. Originally, ×125.

absorbing mantle." Without melanin pigmentation of the skin, man could not tolerate exposure to the skin without fear of excessive sunburn. Malignant melanomas arise from melanocytes and can be pigmented or melanotic due to the massive accumulation of melanin, which imparts an intense black coloration to these lesions. Due to the fact that some melanocytes may be less well-differentiated and therefore produce less melanin, these malignant lesions may be nonpigmented or amelanotic.

Our study shows that human xenograft "nonmelanin pigmented" amelanotic melanoma is highly sensitive to PDT with HpD. Full thickness 100% tumor necrosis was observed following doses of HpD-PDT consisting of 10 mg/kg of Photofrin II and 630-nm red light treatments of 75 J/cm^2. Melanotic melanoma, which contains large quantities of the competing chromophore melanin, shows only superficial necrosis to a depth of 2-3 mm below the tumor surface by use of the same curative treatment parameters for amelanotic melanoma. Even with incident doses of light as high as 200-300 J/cm^2, small areas of viable tumor cells still persisted at the peripheral extremes of the tumor.

Using the methodology described by Kessel (*7*), we found that the total porphyrin accumulation of the melanotic melanoma 24 hours after injection of Photofrin II, as described above, was on the average 8.42 μg porphyrin/g tumor tissue. This was approximately 1 μg more porphyrin than was found to be present in the amelanotic melanoma (7.44 μg/g tumor tissue). This result would exclude the possibility of different tumor cell kinetics, such as uptake and retention of porphyrin as being the reason why amelanotic melanoma tumor responded well and melanotic melanoma did not.

Our study suggests that the presence of the melanin in melanotic melanoma will result in inefficient phototoxicity during PDT treatments. Protection by melanins against photodamage in model systems can occur by several mechanisms. Melanin may compete with the porphyrin for the absorption of photons or in the energy transfer process from the excited triplet state of the sensitizer to melanin instead of cellular oxygen. Furthermore, at least one study has suggested that melanin may in fact be a very effective quencher of singlet oxygen in aerobic photosensitization (*13*).

Extrapolation of the results to the human patient does involve some uncertainties, but further study on PDT with amelanotic melanoma seems promising and warranted. From a practical standpoint, PDT of melanotic melanoma may be useful in the elderly and debilitated patient who cannot tolerate extensive conventional surgery as an adjuvant to chemotherapy, immunotherapy, and radiotherapy. However, the risks at present of leaving viable tumor behind after PDT seem too high for patients who are operative candidates.

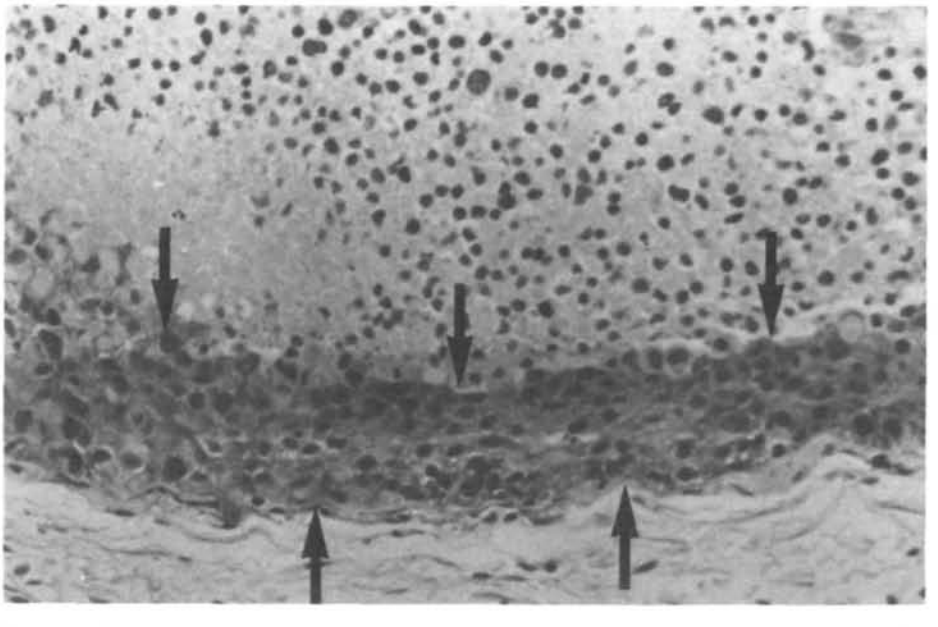

Figure 4. Photomicrograph of human xenograft melanotic melanoma tumor removed 24 hr after treatment with Photofrin II and 300 J/cm^2 of light. Note persistent viable nests of tumor cells between the arrows located at the lateral margin of this tumor. Above is hemorrhagic necrosis of the entire tumor. Below is connective tissue stroma outside of the tumor. Originally, ×210.

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