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Intratumoral Hypericin and KTP Laser Therapy for Transplanted Squamous Cell Carcinoma

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Objectives/Hypothesis: **To test intratumoral photodynamic therapy (IPDT) as a new treatment for squamous cell carcinoma in a preclinical tumor model.** ***Study Design and Methods:*** **Human P3 squamous carcinoma cells were transplanted subcutaneously in athymic nude mice and allowed to grow into 300- to 500-mm^3 tumors. Hypericin dye at 1 μg/gm of body weight was injected intratumorally (IT) or intravenously (IV). After 4 hours hypericin biodistribution was assessed in ethanol extracts from tissues by fluorescence spectroscopy. IPDT also was tested by KTP laser fiberoptic insertion in tumors 4 hours after IT dye injection compared to KTP532 laser therapy alone (532 nm, 1W, 40–60 J, 0.6-mm fiber).** ***Results:*** **Hypericin concentration in tissues was as follows: (IT vs. IV) for tumors (3660 vs. 135 ng dye/gm tissue), lung (760 vs. 6345), liver (75 vs. 935), blood (65 vs. 480) compared to skin (465 vs. 110) or muscle (335 vs. 80) adjacent to the squamous cell tumors. Four hours after dye injection, the tumor exhibited bright orange fluorescence when excited by KTP 532-nm green laser light. The IPDT-treated tumors had a 3.32 ± 0.32-mm radius of cell destruction when H&E-stained sections were examined compared with 2.5 ± 0.38 mm for the laser only control group (n = 10, *P* = .003).** ***Conclusions:*** **This pilot study indicates laser IPDT with hypericin induces a significant increase in tumor necrosis compared with laser alone and may be useful as a less invasive adjuvant treatment for recurrent or inoperable human squamous cell cancers of the head and neck.** ***Key Words:*** **Phototherapy, hypericin, interstitial laser fiberoptics, squamous cell carcinoma.**

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INTRODUCTION

Photodynamic therapy (PDT) is an adjunctive modality for treatment of cancer that is useful in ablation of superficial malignancies. In PDT a light-activated photosensitizer is administered, resulting in the production of toxic oxygen species. Clinical use of PDT has been limited by poor light penetration and low photosensitizer levels within larger tumors. Laser thermal energy delivered by imaging-guided fiberoptics is also being tested as a more effective treatment for a number of inoperable cancers. Palliation by interstitial laser therapy (ILT) is under evaluation as a useful alternative to surgical excision for the ablation of metastatic breast and colon tumors as well as advanced or recurrent squamous cell cancer of the head and neck.[1,2] One way to improve outcome in larger or invasive tumors might be to combine ILT with PDT.

Among the most active new photosensitizers is hypericin, a lipophilic dianthraquinone isolated from herbal extracts of the plant St. John's wort. Hypericin contains extended pi-orbital electrons that are excited by ultraviolet or visible light, leading to production of singlet oxygen as well as protons and radical species, which results in phototoxicity even under mildly hypoxic conditions.[3] Confocal laser fluorescence microscopy has revealed initial hypericin uptake in tumor cell membranes and mitochondria with later migration into the nucleus.[4] Several groups have reported that photoactivation of hypericin at nanomolar levels inhibits protein kinases, blocks growth factors, and induces tumor cell apoptosis.[5,6] Hypericin photoexcitation also causes oxidative stress in mitochondria and decreases ATP synthesis, leading to programmed cell death.[7]

Human tumor transplants in nude mice have been eliminated by phototherapy with 150 J/cm^2 of white light 2 hours after intraperitoneal hypericin injection.[8] Chung et al.[9] previously reported elevated hypericin uptake in

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tumors compared with adjacent normal tissues including skin and muscle 4 hours after intravenous injection of dye into human squamous cell carcinoma–bearing nude mice. Recent clinical testing has revealed that hypericin was not tumoricidal in a patient with mesothelioma exposed to 632-nm red laser light, but clear evidence of phototoxicity was seen in two subjects after intradermal drug injection when activated by green laser emissions at 514 nm rather than by 632-nm or 670-nm red light.[10,11]

These preclinical and clinical in vivo studies with hypericin in human tumors are encouraging but require further exploration, especially in defining drug dose, route of injection, and light delivery for optimal phototherapy responses. Taken together, these initial hypericin experiments suggest that improved tumor responses are likely if careful selection of laser excitation wavelength is coupled with optimal drug dose and route of injection. The current study was performed after intralesional injection of the dye in human tumor transplants in an attempt to increase tumor sensitization and decrease normal tissue uptake as well as to improve light delivery via intratumor laser fiberoptics.

MATERIALS AND METHODS

Tumor Transplantation

UCLA-P3 human squamous cell carcinoma cultures were grown in Costar T75 flasks, removed from monolayers with trypsin, and counted. The P3 cell line was established in 1975 at UCLA from a pulmonary squamous cell carcinoma of a 53-year-old white male patient. The P3 cells were grown as monolayers in a 5% CO_2 incubator in RPMI 1640 media supplemented with 10% fetal calf serum, L-glutamine, and antibiotics. These P3 cells (10^7 cells/0.1 mL) were injected subcutaneously into 6- to 8-week-old BALB/c athymic nude mice and maintained in the UCLA Johnson Cancer Center breeding colony. After 3 to 4 weeks, the squamous cell carcinoma tumors reached volumes ranging from 300 to 500 mm^3 when measured by vernier calipers. Volume was calculated by the formula: $V = \pi/6\ (L \times W \times H)$. During therapy experiments, the mice were housed in filter-top sterile cages under subdued light and fed germ-free food and water. This human tumor transplantation technique in nude mice and the experimental protocols listed below received approval by the UCLA Animal Research Committee following current National Institutes of Health guidelines.

Hypericin Biodistribution and Quantitative Fluorescence

Hypericin was obtained from Roth (HPLC grade, Karlsruhe, Germany). As shown in Figure 1, hypericin has a dianthraquinone structure that absorbs visible light at 545 nm and 590 nm with fluorescence emission maximums at 595 nm and 640 nm. Hypericin stock solutions at 2 mg/mL in 95% ethanol were diluted immediately before use in normal saline. Hypericin (1 μg/g of body weight) was injected transcutaneously into the tumor center or intravenously via lateral tail vein using a 0.3-mL syringe with a 30-G needle. Four hours after injection, mice were sacrificed and tumors, blood, lung, liver, skin, and muscle were harvested. Blood was obtained by cardiac puncture and sera were collected after centrifugation. Skin and muscle were obtained from areas adjacent to tumor. The specimens were weighed and stored in airtight containers at 4°C until ethanol extraction and homogenization were performed. Each specimen (100 mg) was diluted in 10 to 50 volumes of 95% ethanol and homogenized with a BioSpec Tissue-Tearor (Bartlesville, OK), tubes were centrifuged for 5 minutes at 2400 rpm, and supernatants were stored in the dark. Sera were diluted 20-fold with 95% ethanol before centrifugation and supernatants were stored at 4°C in the dark before measurement.

Fluorescence measurements were carried out in a spectrofluorometer (Fluoromax, Spex Industries, Edison, NJ) containing a 150-W xenon light source and a 1681 excitation monochronometer driven by DM3000 computer software. The excitation wavelength was selected at 588 nm, with the fluorescence emission maximum of 640 nm (Fig. 1) measured to quantify hypericin in ethanol extracts. Fluorescence measurements were performed on sample supernates with emission peak intensities recorded as counts per second (CPS) and converted to ng dye/gm tissue using 1 to 1000 ng/mL hypericin in ethanol as calibration standards. Adding known dye levels before homogenization showed that extraction efficiency was more than 95% for most biopsied samples but less than 75% for skin specimens.

Interstitial Laser Therapy, Photodynamic Therapy, and Histopathologic Evaluation

Five human squamous cell carcinoma control tumors in nude mice were treated by ILT after ketamine/xylazine intraperitoneal anesthesia using a KTP laser (Laserscope, San Jose, CA.) emitting continuous wave 532-nm green light. During laser operation the 600-μm bare fiberoptic was inserted vertically through the skin into each tumor and moved down to the tumor base slowly over 40 to 60 seconds. The power setting of the KTP laser was 1 W, with total energy delivery of 40 to 60 J adjusted in proportion to 300 to 500 mm^3 tumor size. Hypericin (20 μg/50 μL) was injected directly into the heterotransplanted squamous cell carcinoma tumors in nude mice (n = 5). Four hours after intratumor injection of hypericin, the entire tumor exhibited a bright orange-colored fluorescence when excited by the KTP532 laser and viewed through goggles to block the exciting green 532-nm laser light. At this point the squamous cell carcinoma tumors were treated by IPDT via laser fiberoptic insertion as described above. Tumor specimens were harvested 48 hours after laser treatment and fixed in formalin for at least 2 days. These specimens were completely dehydrated in graded water alcohol mixtures and embedded in paraffin. The paraffin-embedded tumor

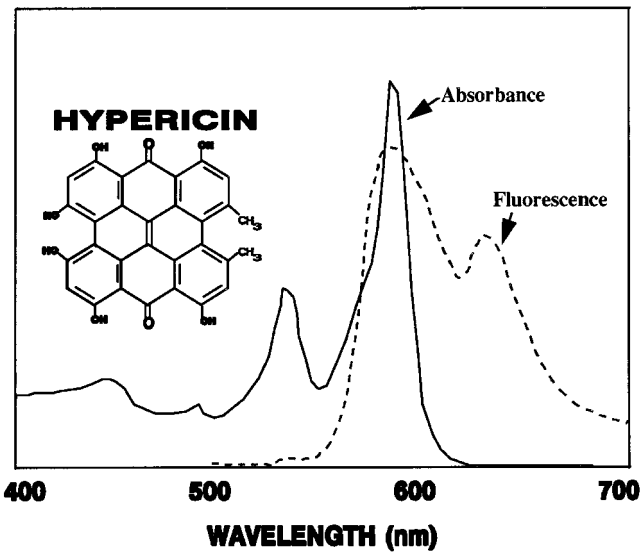

Fig. 1. Hypericin dianthraquinone structure and visible light excitation/fluorescence spectra.

specimens were sectioned parallel to the vertical fiberoptic insertion axis before being stained with H&E. Sections of each specimen were examined by a light microscope to measure the extent of tumor necrosis extending outward from the KTP laser bare fiberoptic insertion track. The H&E-stained sections were viewed at magnification × 12 and three separate measurements were performed to define a mean radius of necrosis for each tumor in the ILT and IPDT groups (30 total measurements).

RESULTS

Biodistribution of Hypericin

The first experiment was to measure dye levels in tumor and tissue extracts. Figure 2 compares the hypericin dye uptake seen 4 hours after intratumor versus intravenous injection. Hypericin injected intravenously in squamous cell carcinoma–tumor bearing mice led to very high dye levels at 4 hours in lungs (6350 ng/gm of tissue) with elevated uptake in liver (935 ng/gm) and blood (480 ng/gm), whereas tumors (135 ng/gm), skin (110 ng/gm), and muscle (80 ng/gm) had much lower concentrations. By contrast, 4 hours after intratumor injection human squamous cell tumor transplants retained the highest hypericin uptake (3660 ng/gm), as shown in Figure 2. Tissues adjacent to tumor had clearly elevated dye levels, with skin containing 465 ng/gm and muscle 335 ng/gm. This dye uptake level was fivefold higher than in the same tissues seen after intravenous injection. Lung uptake (760 ng/gm) was 15-fold reduced after intratumor injection compared with intravenous injection, whereas liver (75 ng/gm) and blood (65 ng/gm) contained 10-fold lower dye levels, as shown in Figure 2.

Histopathology of Tumor Response

A bright orange-colored fluorescence was observed in the tumor center at the dye injection sites when excited by green 532-nm light. This orange hypericin fluorescence spread throughout the tumor with increased time after dye injection but remained largely localized in the tumors as shown in Figure 3. A noticeable increase in this fluorescence intensity was observed soon after interstitial fiberoptic insertion and KTP laser illumination, suggesting that IPDT induced immediate tumor cell membrane damage and enhanced intracellular dye diffusion. Microscopic examination of the KTP laser fiberoptic–induced lesions in tumors by H&E staining after ILT or IPDT revealed a central zone of tissue destruction and vaporization surrounded by a zone of coagulative necrosis, in turn surrounded peripherally by a zone of pallor as shown in Figure 4. The ILT group (n = 5) had a cell necrosis mean distance of 2.5 ± 0.38 mm from the laser fiber insertion axis and the IPDT tumor group (n = 5) had a 3.32 ± 0.32-mm radius of cell destruction from the fiberoptic axis. The difference was statistically significant ($P = .003$) by unpaired t test, indicating that IPDT with hypericin led to a measurable increase in tumor ablation.

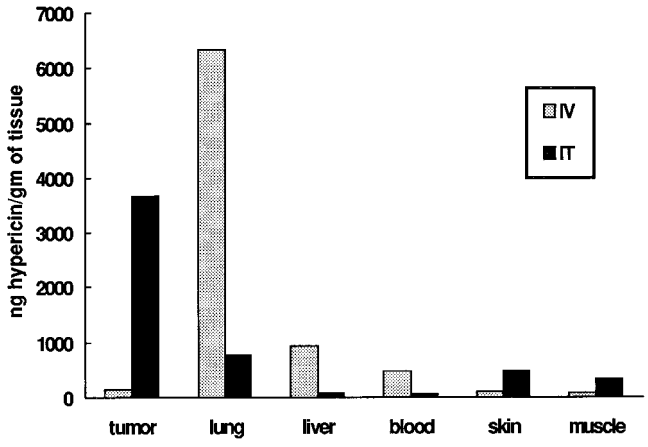

Fig. 2. Hypericin biodistribution 4 hours after intratumor (IT) versus intravenous (IV) injection.

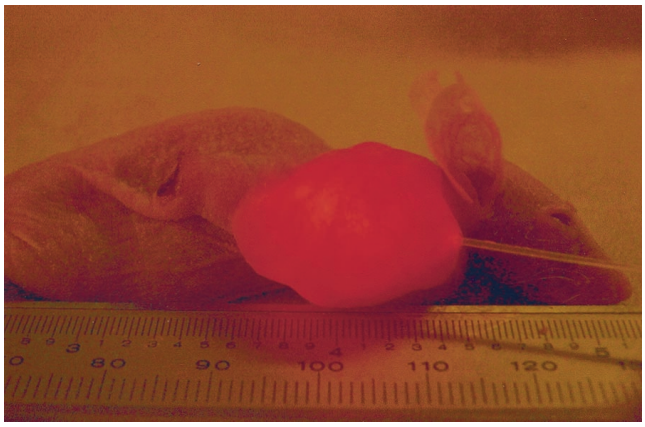

Fig. 3. Laser-induced hypericin fluorescence in squamous cell tumor 4 hours after intratumor injection.

DISCUSSION

Laser fiberoptic ablative surgery under the guidance of magnetic resonance imaging or ultrasonography has been tested as a less invasive alternative than conventional surgical excision for tumor palliation in brain, prostate, breast, liver, and head and neck malignancies. In some cases ILT can be performed in the clinic under local anesthesia and may be repeated at intervals to increase the tumor response with less morbidity and cost than conventional surgery.[2] One way that may improve tumor response is to inject light-sensitive anticancer drugs into tumors before laser irradiation via fiberoptics.[12] Vanderwerf et al.[13] first reported strong phototoxicity of the KTP laser and hypericin in human tumor cell lines.

In the current in vivo study hypericin was injected directly into human squamous cell tumor transplants in mice. The dye was activated by KTP532 laser light delivered via fiberoptics, leading to enhanced ablation and intense orange-colored dye fluorescence throughout the tumors. Gamache and Morgello[14] have described histopathologic tissue changes induced by KTP laser fiberoptics including a central zone of tissue destruction and vaporization surrounded by a zone of coagulative necrosis enclosed in turn by an outer zone of pallor. Although the wavelengths of the KTP532 laser and argon 514-nm laser are similar, tissue interactions due to the KTP laser are much less pigment dependent.[14] Histopathologic observa-

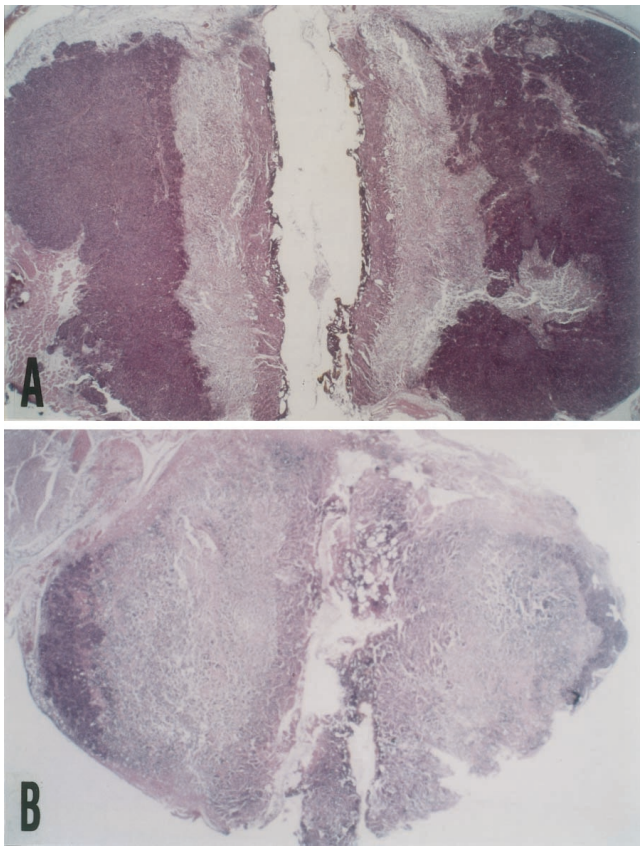

Fig. 4. Histopathology (original magnification ×12) of laser-induced damage in tumors after interstitial laser therapy **(A)** versus a broader area of densely stained necrosis extending from the fiberoptic axis seen after interstitial photodynamic therapy **(B)**.

tion of the specimens treated by IPDT in the present study revealed that hypericin-sensitized tumors contained a significantly wider area of cellular destruction than after ILT alone.

Vandenbogaerde et al.[8] previously reported that hypericin uptake and fluorescence were enhanced in tumors after light exposure, suggesting that increased tumor permeability may have been induced by PDT. Interestingly, in the current study an increase in hypericin fluorescence intensity also was seen when the tumor was illuminated by KTP laser light. A 4-hour interval between intratumor dye injection and KTP laser therapy led to visible hypericin uptake, with orange fluorescence rapidly diffusing throughout the transplanted squamous cell tumors. This intralesional injection resulted in 25-fold higher tumor retention of hypericin at 4 hours compared with systemic injection and 10-fold less dye in lungs, but there were increased dye levels in skin and muscle adjacent to tumors.

Clearly visible dye diffusion in tumors is likely to be a major advantage for surgeons before and during laser IPDT. Even at 4 hours after injection, minimal hypericin fluorescence was detected beyond the squamous cell carcinoma tumor margins. This observation indicates that intratumor dye injection may provide real advantages for both tumor localization and decreased normal tissue phototoxicity compared with conventional systemic dye administration. Intratumor KTP532 laser fiberoptic delivery of green light with limited penetration capability into adjacent tissues also allows precise surgical control to monitor intraoperative fluorescence and avoid normal tissue phototoxicity. Cylindrical diffusers have been reported to increase both treatment volume and lateral light distribution by laser fiberoptics for tumor therapy.[15] The recent development of diode and fiberoptic lasers allows use of deeply penetrating near infrared light with photosensitizers. One or more of these approaches may lead to a more effective phototherapy for patients with head and neck cancer.

CONCLUSION

A new approach for photodynamic therapy was tested using direct intralesional injection of hypericin before interstitial KTP532 laser fiberoptic insertion and illumination of human squamous cell tumors transplanted in athymic nude mice. Hypericin dye fluorescence was visible via laser illumination immediately after injection and rapidly diffused to the margins of the squamous cell carcinoma tumors. A much higher concentration of hypericin was retained in the tumors 4 hours after intralesional dye administration compared with intravenous dye administration. Significant photoactivation of hypericin by the KTP laser fiber resulted in an increased radius of tissue destruction in tumors treated by IPDT versus ILT when compared by histopathology. This intratumor phototherapy method must be tested further before optimal drug dose, route, timing, and laser dosimetry can be defined for clinical evaluation as an adjuvant therapy in head and neck cancer patients.

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