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Photodynamic Therapy of Cottontail Rabbit Papillomavirus-Induced Papillomas in a Severe Combined Immunodeficient Mouse Xenograft System

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Abstract

Objectives/Hypothesis—To evaluate the efficacy of photodynamic therapy (PDT) with the phthalocyanine photosensitizer Pc 4 for treating an animal model of recurrent respiratory papillomatosis (RRP).

Methods—Rabbit skin was grafted onto the dorsum of severe combined immunodeficient mice, two xenografts per animal. After the graft healed, it was inoculated with cottontail rabbit papillomavirus (CRPV). When papillomas developed, Pc 4 (0.6 or 1.0 mg/kg) was administered systemically, and 48 hours later, one papilloma of the two on each animal was exposed to 675-nm photoactivating light at either 100 or 150 J/cm². In addition to the contralateral tumors, which received Pc 4 but no light, other controls included animals receiving light only or neither agent. Response was assessed by measuring papilloma size with a caliper. Some papillomas and residual skin were harvested for histological assessment.

Results—For the lower-dose PDT regimens, papilloma growth rates were not significantly different from the controls. In contrast, 13 of 15 papillomas receiving the higher Pc 4 dose (1.0 mg/kg) and the higher light fluence (150 J/cm²) regressed completely and did not regrow within the observation period of up to 79 days. The response of these papillomas was significantly different from the controls ($P < .001$). Histological analysis confirmed the absence of residual tumor following complete response and replacement with near-normal epithelium.

Conclusions—Pc 4-PDT is highly effective in treating virally induced (CRPV) papillomas in a murine model of RRP, and thus warrants further study as a treatment for HPV-induced papillomas.

Keywords

Recurrent respiratory papillomatosis; photodynamic therapy; xenograft; phthalocyanine

INTRODUCTION

Recurrent respiratory papillomatosis (RRP) lacks a satisfactory treatment modality. Surgical removal is the therapeutic mainstay with goals that include maintenance of the airway and preservation of vocal quality. Recurrence often requires patients to return for re-excision, sometimes as often as every 4 weeks to maintain a stable airway.¹ Many nonsurgical adjunct therapies, including the use of interferon alpha, acyclovir, ribavirin, indole 3-carbinol, and intralesional injection of cidofovir, have been studied in an attempt to minimize or even eliminate the need for surgical intervention.¹ Medical therapies to date have not eliminated the need for surgery and remain adjunctive to surgical removal. Human papillomavirus (HPV) vaccines are US Food and Drug Administration (FDA)-approved to prevent cervical cancer, low-grade and precancerous lesions, and genital warts caused by HPV types 6, 11, 16, and 18. Use of this vaccine may impact future treatment of RRP; however, the FDA does not currently approve the vaccine for this indication. Moreover, the vaccines are thought to be preventative and not therapeutic.² Further research to explore novel treatments of RRP is warranted.

Photodynamic therapy (PDT) was originally developed for the diagnosis and treatment of cancer with a presumed advantage of focused tumor cell killing without damage to normal cells.^{3,4} Porfimer sodium (Photofrin; Axcan Pharma, Birmingham, AL) is an FDA-approved photosensitizer for use in lung cancer, esophageal carcinoma, and Barrett's esophagus. RRP is a good candidate for PDT, as the disease leads to rapidly dividing cells that occur in a superficial anatomic location. PDT has been explored as a treatment option for RRP for over a decade. Initial results were overshadowed by the long-term cutaneous photosensitivity (approximately 2 months) associated with Photofrin. A newer photosensitizer, m-tetra hydroxyphenyl chlorin (Foscan; Biolitec Pharma, Dublin, Ireland) has also been tested against RRP. Initial results of Foscan PDT in RRP were encouraging, reducing the tumor size and recurrence rate of papillomas.⁵ Foscan was approved for use in Europe for certain cases with advanced head and neck squamous cell carcinoma,⁶ but it was not approved by the US FDA in 2000. Foscan has a very long residence time in tissue, such that patients can remain photosensitive for a longer time than with Photofrin, and the phototoxic responses can be very intense for 2 weeks or longer.^{7,8} Photosensitizers that confer less severe photosensitivity while providing clinical efficacy are needed.

Pc 4 is a silicon phthalocyanine developed and studied at Case Western Reserve University.⁹ Pc 4-PDT targets mitochondrial and endoplasmic reticulum membranes, activating cellular pathways of apoptosis through the generation of reactive oxygen species. Pc 4 exhibits lower phototoxic side effects than Photofrin in experimental animals, including a much shorter period of cutaneous photosensitivity with milder and more rapid resolution of phototoxicity in normal skin.¹⁰ In a limited study of patients receiving Pc 4 intravenously in a phase I dose-escalation trial for dermal cancers, no cutaneous photosensitivity was

observed outside the treatment area (unpublished data). In addition, Pc 4 is activated at a longer wavelength of light ($\lambda_{\text{max}} = 670\text{--}675\text{ nm}$), allowing for greater depth of light penetration into tissue than for Photofrin and most other photosensitizers currently in use. Pc 4 is much more photostable than other agents and has increased fluorescence (and thus photoactivity) during irradiation of cells—a property that may be useful for monitoring the delivered dose of PDT with Pc 4 within tissue.¹¹

Papillomas have been successfully produced on human foreskin grafted onto severe combined immunodeficient (SCID) mice through inoculation with HPV-16 DNA.¹² However, studies employing HPV to induce tumor growth can be problematic, requiring extensive biosafety hazard precautions and expense. Cottontail rabbit papillomavirus (CRPV) has significant biosafety advantages, because it exhibits host selectivity to rabbit epithelium only. In addition, it has extensive genetic homology with HPV. CRPV-related animal models have been used extensively to study the pathogenesis and treatment of HPV-related diseases. Shikowitz first studied PDT in a rabbit CRPV model in 1986, and subsequently extended these investigations to treatment of RRP in clinical trials.^{5,13,14}

CRPV is selective for cutaneous tissue. In contrast, RRP occurs in response to activated HPV infection of respiratory mucosa. A rabbit oral papillomavirus (ROPV) that induces papillomas in mucosal tissues has been described. However, regression occurs in 100% of ROPV-induced papillomas, making assessment of therapeutic intervention difficult.¹⁵ In addition, conducting large-scale studies of PDT on oral papillomas of rabbits would be cumbersome and costly. An easily reproducible murine model is more practical.

A CRPV xenograft model was first described by Kreider where rabbit epithelium grafted onto immunodeficient nude mice was inoculated with CRPV.¹⁶ PDT studies on CRPV-induced papillomas in a SCID mouse model have not previously been reported. Using this model, the efficacy of Pc 4-PDT on CRPV-induced tumors was investigated.

MATERIALS AND METHODS

All animal studies were approved by the Case Western Reserve University School of Medicine Institutional Animal Care and Use Committee (IACUC). New Zealand White rabbits purchased from Covance Research Products Inc. (Princeton, NJ) and SCID mice (colony of BALB/c-Igh^b [C.B.-17]) purchased from The Jackson Laboratory (Bar Harbor, ME) were housed in the Athymic Animal Facility at Case School of Medicine and utilized in the study.

The procedure used in this report is outlined in Figure 1. Briefly, full-thickness skin grafts were harvested from the back of the ears of the rabbits. A 7-mm punch biopsy forceps separated the harvested skin into individual grafts. The xenografts were then placed onto reciprocal 7-mm wounds on the backs of C.B.-17 SCID mice, as described by Lobe et al.¹⁷ Two grafts were placed per mouse and secured with tissue adhesive and a circumferential bandage. All xenografts were monitored for shrinkage and/or failure. After allowing approximately 3 weeks for the grafts to heal, the transplanted epithelium was inoculated with undiluted thawed CRPV suspension, and 4 μL of the undiluted CRPV stock was placed

on the xenograft, which was then scarified through the solution with a 27-gauge hypodermic needle about 100 times. The scarified graft was reinoculated with an additional 4 μ L of the CRPV stock. This procedure was repeated for the contralateral graft. Cutaneous papillomas were allowed to grow to a score of 5, as described by Syverton et al.,¹⁸ which required approximately 4 weeks. At this stage, the tumor was approximately 3-mm to 6-mm thick. Mice with active papilloma growth were divided into five subgroups for dosing with Pc 4 and light (Table I; Fig. 1). The Pc 4 and light doses were based on previous studies of Pc 4-PDT on solid malignant tumors conducted at Case Western Reserve University.^{19–22}

Pc 4 (0.6 or 1.0 mg/kg) was administered intravenously via tail-vein injection. An interval of 48 hours was allowed between Pc 4 injections and photoirradiation to allow for washout of Pc 4 from normal tissues. During that interval, animals were protected from ambient light. A diode laser delivering light at a wavelength of 675 nm was used to irradiate the grafts with 100 or 150 J/cm² at an irradiance of 75 mW/cm². Only one papilloma on each animal was irradiated, and the contralateral graft served as a Pc 4-only control.

The papillomas were measured in three dimensions approximately every 7 days with a microcaliper rounding to the nearest millimeter. The volume of the papillomas was calculated using the formula $4/3\pi \times \frac{1}{2} \text{ length} \times \frac{1}{2} \text{ width} \times \frac{1}{2} \text{ height}$ and recorded. Initially, a total of 26 animals (52 grafts) were studied. To increase the statistical power, an additional 11 animals (22 grafts) were added at a later date.

Statistical Analysis

Tumor volumes were expressed in cubic centimeters. Comparisons of tumor growth rates between treatments (Table I) were made by fitting a linear regression of $\ln(\text{volume} + 1)$ versus post-treatment days for each tumor to obtain the slope of the regression for each tumor. The constant 1 was added to the volume before taking logarithms to avoid taking the logarithm of 0. The mean slopes of the different treatment groups were then compared to the mean slope of the controls (i.e., a combination of tumors exposed to Pc 4 only [contralateral tumors], or tumors on other mice exposed to light only, or neither). These analyses used a mixed model with animal as a random effect, thereby controlling for the fact that tumors from the same animal are not independent. The Bonferroni method was used to control for multiple testing. Based on four comparisons of treatments versus control, differences were considered significant at the .05 significance level if the *P* value was less than .0125.

Not all animals and tumors were included in the analysis. Grafts with 0 volumes were excluded from the analysis. The initial set of experiments selected only tumors with volumes between 0.075 and 0.38 cm³ at the time of PDT. Note that in the second set of experiments this size restriction was not enforced, and in fact all tumor volumes were less than 0.075 cm³ at baseline. Among 26 animals in the first set of experiments, four had both grafts excluded, and nine others had a single graft excluded because the graft failed to meet the baseline volume requirement. This resulted in 35 grafts from 22 animals. Among 11 animals in the second set of experiments, one animal died before any measurements could be obtained after baseline, and six unilateral grafts with 0 volume at baseline and all follow-up times were excluded, resulting in 14 grafts from 10 animals. The entire data set, therefore, contained 49 grafts from 32 animals. To control for possible differences in growth due to the different

inclusion criteria of the two sets, experimental set (first vs. second set) was included as a stratification factor in the analysis (see Table I).

RESULTS

Overall xenograft success rate, as defined by maintenance of size and configuration of the graft at 3 weeks, was 83.9%. Initial graft failure rates were approximately 25%. However, improvement in bolster technique led to greater protection of the graft and limitation of animal-produced shear forces. This improved the xenograft success rate to approximately 95% in subsequent animals in the initial data set. Figure 2A shows rabbit epithelium xenografts on the back of a SCID mouse at approximately 2 weeks post-transplant. The photograph demonstrates maintenance of graft size and configuration bilaterally. Figure 2B demonstrates full integration of the xenografts at about 10 weeks post-transplantation.

The papilloma-induction rate approached 84%. We defined papilloma induction as confluent papilloma growth, which correlates to a score of 5 as described by Syverton et al.¹⁸ and Lobe et al.¹⁷ Of the control (noninfected) animals, no adverse cutaneous outcomes were observed in animals treated with Pc 4 or laser irradiation alone. The noninfected animals that underwent PDT of the xenograft had a fatal outcome, regardless of the dosing regimen. This was likely a result of injury to vital internal organs lying under the xenograft that were not shielded by a 3-mm- to 5-mm-thick papilloma. Lethal photodynamic damage due to light penetration well into the mouse's body is recognized to be a concern during PDT of small animals.²³

The CRPV-inoculated animals showed rapid growth of their papillomas starting around 20 days postinoculation, progressing to large cutaneous warty lesions with dense keratinous horns. There was no difference in the pattern or rate of growth of papillomas in animals treated with Pc 4 only (Fig. 1) in comparison to papillomas in animals that were not injected with the photosensitizer (Fig. 3A).

The results of Figure 3A indicate that tumor growth was markedly reduced in the group receiving 1.0 mg/kg Pc 4 plus 150 J/cm² laser light. This was confirmed by comparison of mean slopes of Ln (tumor volume+1) versus time in the mixed model analysis; the 1.0 mg/kg plus 150 J/cm² laser light group had a lower mean slope ($P = .0004$) compared to the control tumors. The mean slopes of each of the other three treatment groups did not differ from the control mean slope ($P > .20$). Similar findings resulted when data from the first and second experiments were analyzed separately. Notably, of the 15 tumors treated with this combination of Pc 4 and light, 13/15 (87%), 4/5 from the first experiment and 9/10 in the second experiment ($P = \text{NS}$), completely regressed to 0 volumes, whereas 0/34 tumors given the other treatments showed complete regression.

The time course of response of the 15 individual tumors in the group given 1.0 mg/kg Pc 4 plus 150 J/cm² is shown in Figure 3B. In this case, tumor size post-treatment is expressed as a percentage of its size at the time of treatment (week 0). It is apparent that 13 tumors regressed to 0 volume but with varying kinetics. A few of the tumors increased in size

before eventually shrinking. Also shown are the two tumors that did not respond completely and eventually regrew.

Papillomas that regressed after treatment with 1.0 mg/kg Pc 4 and irradiation with 150 J/cm² demonstrated normal xenografted epithelium on gross inspection. Animals were examined weekly; the epithelium showed no evidence of recurrence up to 79 days following treatment. Figure 4 is a photograph of one of the animals treated with 1.0 mg/kg Pc 4 and unilateral irradiation with 150 J/cm² of light; it was evident that the treated tumor regressed completely, in contrast to the nonirradiated one.

Histological analysis confirmed the gross examination. Cytologic features of virus-induced tumor growth were not present in the specimens from regressed tumors. However, scattered cellular changes consistent with early viral infection were noted in some of the specimens at the time of harvest, which confirms that the transplanted epithelium has remained viable because CRPV only infects rabbit tissue. Figure 5 demonstrates representative histology from an untreated papilloma.

Figure 6 shows tissue sampled from a xenograft whose induced papilloma had totally regressed following PDT. Close examination shows normal keratinizing squamous epithelium with focal cellular changes consistent with early viral infection.

DISCUSSION

The use of a photoactive substance to destroy tumor cells with light activation has a long history. Pc 4-PDT has been studied extensively in murine models for solid malignant tumors. Previous work explored the utility of Pc 4 in immunodeficient nude mice implanted with human squamous cell carcinoma.¹⁹ Xenografts of human glioma,²⁰ colon,²¹ ovarian,²² breast,²⁴ and prostate²⁵ tumors have also been studied with Pc 4-PDT at Case Western Reserve University with excellent tumor responses and little or no evidence of damage to the surrounding normal tissues.^{20–22} Pc 4-PDT is currently being tested in a phase I clinical trial of cutaneous malignancies. An in vitro study demonstrated preferential killing of virally infected cells by Pc 4-PDT.²⁶ However, the efficacy of Pc 4-PDT in treating virally induced benign tumors has not been tested.

In this article, we report initial data on the effects of Pc 4-PDT on CRPV-induced tumors. We found that with sufficient Pc 4 and light, 87% of CRPV-induced tumors regressed and did not recur up to 79 days post-treatment. Notably, the effective dose of Pc 4-PDT was within the range found to be effective against solid malignant tumors in mice. These results are encouraging, but further experimentation is necessary to establish the lowest effective dose of Pc 4 for treatment of CRPV-induced tumors.

A study with a longer observation period monitoring for papilloma regrowth is prudent considering the cellular changes detected on high-power light microscopy. Shikowitz et al.¹³ reported on the use of hematoporphyrin derivative for treatment of CRPV-induced papillomas on rabbits. They observed animals up to 6 months post-treatment and found no recurrence during this period.¹³ If further study with the SCID mouse xenograft model

reveals earlier recurrence than with rabbits, this may indicate that an intact immune system is required for optimum tumor control.^{27–29}

Published reports suggest that the beneficial effects of PDT in the treatment of RRP may be due in part to alteration of the immune response to viral proteins. Recently, Shikowitz et al. reported on the use of metatetra hydroxyphenyl chlorine (m-THPC) in clinical trials of RRP patients.⁵ They measured the concentration of E6, an HPV protein, required to induce IL-10 and IFN- γ messenger RNA expression in peripheral blood mononuclear cells from patients undergoing PDT. They correlated the disease severity with the concentration of E6 required to induce these cytokines. Based on this analysis, they suggested that the efficacy of PDT with m-THPC reflects an improvement in the immune response by indirectly immunizing the patient to HPV proteins.

The SCID mouse has no T or B cell activity, and therefore cannot mount an immune response. Applying the theory that PDT is most effective when it stimulates the immune system, we would expect decreased efficacy of treatment with a higher recurrence rate in our model. Our initial findings suggest that the immune response is not critical in early tumor ablation, but may play a role in longer-term tumor control. In this regard, response of benign papilloma growths may be similar to responses of solid malignant tumors in mice.^{28,29} A next step in evaluating Pc 4-PDT may include limited trials in cottontail rabbits, similar to Shikowitz's initial studies. Using animals with an intact immune system may demonstrate improved efficacy and decreased recurrence rates. Comparing the SCID xenograft model to one using animals with an intact immunologic system may help elucidate the immune system's role in treatment of papillomas with PDT.

CONCLUSION

Regression of CRPV-induced papillomas occurred in 87% of animals treated with a sufficient dose of Pc 4- PDT (1.0 mg/kg of Pc 4 and 150 J/cm² of light). Our study suggests that Pc 4-PDT may be a potential treatment for HPV-induced papillomas.

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Fig. 1.
Experimental design. SCID = severe combined immunodeficient; CRPV = cottontail rabbit papillomavirus.

Fig. 2. Rabbit xenografts in severe combined immunodeficient mice. Appearance of successful xenografts at approximately 2 weeks (A) and 10 weeks (B) postimplantation.

Fig. 3.

Growth of papillomas and effect of treatments. (A) Growth of papillomas in treatment groups given both Pc 4 and light, and in controls with no Pc 4, or with Pc 4 or laser light only. For each treatment group, the mean volumes $\pm$ one standard error are plotted against post-treatment time. (B) Growth of individual papillomas in treatment group PC1.0L150. The size of each papilloma is expressed as a percentage of its size at time 0. To better show the data, the vertical axis is cut off at 300%. One tumor (line with arrow, asterisk) increased rapidly above 300% after week 4. *Values beyond week 4 are not shown because the animal

that harbored this tumor was euthanized according to protocol at week 7 due to growth of the treated and contralateral tumors above 15% of the body weight of the animal. **This tumor regressed to near 0 but eventually regrew. All others regressed to 0 volume and did not regrow within the 10-week post-treatment observation period.

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Fig. 4. Regression of photodynamic therapy-treated papilloma. The white arrow shows normal epithelium on gross examination after papilloma regression; the black arrow points to a dense keratin horn of a nonirradiated papilloma (Pc 4 only).

Fig. 5. Representative pretreatment photomicrographs of virus-induced papilloma on xenografted severe combined immunodeficient mouse. (A) Low-power hematoxylin and eosin (H&E) stain of papilloma with an overlying dense keratin cap. (B) High-power H&E stain showing typical histological features of robust papilloma growth with diffuse hyperkeratosis and parakeratosis.

Fig. 6. Representative post-treatment photomicrograph of virus-induced papilloma on xenografted severe combined immunodeficient mouse. (A) Low-power hematoxylin and eosin (H&E) stain of thin but normal-appearing epithelial layer following regression of the tumor. Mild stromal changes consistent with skin grafting and photodynamic therapy are observed. (B) High-power H&E stain of normal keratinizing squamous epithelium with focal cellular changes consistent with early viral infection (arrow).

TABLE I

Comparison of Papilloma Growth.

Treatment	Pc 4 Dose (mg/kg)	Fluence (J/cm ²)	N (1st Exp, 2nd Exp)	Mean (SE) of Slope	P Value
Control	0, 0.6, 1.0	0, 100, 150	19 (15, 4)	0.017 (0.002)	
PC0.6L100	0.6	100	5 (5, 0)	0.020 (0.004)	.48
PC0.6L150	0.6	150	4 (4, 0)	0.023 (0.005)	.28
PC1.0L100	1.0	100	6 (6, 0)	0.014 (0.004)	.37
PC1.0L150	1.0	150	15 (5, 10)	0.002 (0.002)	.0004

Table I lists the title of each subgroup of animals, the Pc 4 dose delivered to that subgroup (PC), the fluence to which one of the two tumors was exposed (L), the number (N) of evaluable tumors in each treatment group (and each experiment [Exp]), the mean and the standard error (SE) of the slopes for each group. The *P* values comparing treatment groups to the control are listed. NOTE: The control group consisted of the contralateral tumors from animals in the four treatment groups as well as additional animals that were exposed to light but no Pc 4 or given no treatment. The 19 control tumors consisted of one exposed to neither Pc 4 nor light, one exposed to 150 J/cm² laser light only, seven exposed to 0.6 mg/kg Pc 4 and no light, and 10 exposed to 1.0 mg/kg Pc 4 and no light. The 19 control tumors were in 18 mice (one mouse had neither Pc 4 nor laser light on one side, and laser light only on the other side, and the 17 other control grafts came from 17 different mice).