Enhanced Systemic Immune Reactivity to a Basal Cell Carcinoma Associated Antigen Following Photodynamic Therapy

Edith Kabingu, Allan R. Oseroff, Gregory E. Wilding, and Sandra O. Gollnick

Abstract

Purpose: Numerous preclinical studies have shown that local photodynamic therapy (PDT) of tumors enhances systemic antitumor immunity. However, other than single-case and anecdotal reports, this phenomenon has not been examined following clinical PDT. To determine whether PDT in a clinical setting enhances systemic recognition of tumor cells, we examined whether PDT of basal cell carcinoma resulted in an increased systemic immune response to Hip1, a tumor antigen associated with basal cell carcinoma.

Experimental Design: Basal cell carcinoma lesions were either treated with PDT or surgically removed. Blood was collected from patients immediately before or 7 to 10 days following treatment. Peripheral blood leukocytes were isolated from HLA-A2–expressing patients and reactivity to a HLA-A2–restricted Hip1 peptide was measured by INF-γ ELISPOT assay.

Results: Immune recognition of Hip1 increased in patients whose basal cell carcinoma lesions were treated with PDT. This increase in reactivity was significantly greater than reactivity observed in patients whose lesions were surgically removed. Patients with superficial lesions exhibited greater enhancement of reactivity compared with patients with nodular lesions. Immune reactivity following PDT was inversely correlated with treatment area and light dose.

Conclusions: These findings show for the first time that local tumor PDT can enhance systemic immune responses to tumors in patients, and validate previous preclinical findings.

Photodynamic therapy (PDT) is an antitumor modality that is approved for clinical use in a number of countries, including the United States, for the elimination of early-stage malignancies and the palliation of symptoms in patients with late-stage tumors (1, 2). PDT has traditionally been considered to act locally, causing direct tumor destruction through the generation of reactive oxygen species and acting indirectly through vascular damage (3–5). However, preclinical studies have shown that local PDT treatment of tumors can result in widespread systemic effects that include induction of systemic neutrophilia (6), induction of acute-phase proteins (6, 7), increased circulating levels of complement proteins (8), and systemic release of proinflammatory cytokines (7, 9–13), all of which indicate the presence of a systemic inflammatory response. Subsequent studies showed that local PDT treatment of murine tumors results in resistance to subsequent tumor challenge (reviewed in refs. 14, 15) and in some cases an increased ability to control tumor growth outside the local treatment field (16, 17) that was dependent upon the presence of an intact immune response (17). The general conclusion from these preclinical studies was that PDT leads to the enhancement of antitumor immunity. However, this hypothesis has never been tested in a clinical setting in part due to a lack of known tumor antigens associated with the tumors commonly treated with PDT.

Mutations in the gene for the receptor for sonic hedgehog protein (SHH), patched-1 (PTCH1), are causally involved in the development of basal cell carcinoma (18, 19). Patched-1 negatively regulates the function of smoothen (PTCH1) and the glioma-associated oncogene homolog (GLI) family members, GLI1, GLI2, and GLI3, have all been shown to be up-regulated in basal cell carcinoma (20, 21). Recently a new member of the hedgehog signaling pathway transcription factor family, GLI3, mRNA levels for PTCH1 and the glioma-associated oncogene homolog (GLI) family members, GLI1, GLI2, and GLI3, have all been shown to be upregulated in basal cell carcinoma (20, 21). Currently a new member of the hedgehog signaling pathway gene family has been identified, Hipp (hedgehog-interacting protein; ref. 22). Hipp encodes for type 1 transmembrane protein, Hip1, which binds to all members of the hedgehog family with an affinity similar to that of patched-1 and is thought to have a similar negative regulatory function (22). Hipp is also overexpressed in basal cell carcinoma (20, 21), but it seems to have lower expression in normal skin than PTCH1 and does not seem to be mutated in basal cell carcinoma. Thus, it is possible that Hip1 can act as a tumor-associated antigen...
was confirmed using a modification of the T2 stabilization assay (25). mL) of peptides were made in RPMI-1640. Peptide binding to /C0 DMSO and stored at a temperature facility. Stock solutions of peptides were made in 100% synthesis at the University of Georgia Molecular Genetics Instrumentation facility. Identification of HLA-A2–binding peptides. The Bioinformatics and Molecular Analysis Section (BIMAS) of the NIH (Bethesda, MD)2 was used to identify Hip1 peptides with HLA-A2–binding potential. BIMAS calculates a “theoretical” binding stability matrix based on the rate of dissociation from β2-microglobulin (β2M), which was developed by Parker et al. (24), to predict potential HLA-A2–binding peptides. Potential HLA-A2–binding Hip1 peptides were synthesized at the University of Georgia Molecular Genetics Instrumentation facility. Stock solutions of peptides were made in 100% DMSO and stored at −20°C until use. Working concentrations (500 μg/mL) of peptides were made in RPMI-1640. Peptide binding to HLA-A2 was confirmed using a modification of the T2 stabilization assay (25). Briefly 106 T2 cells were incubated in RPMI-1640 in the presence of peptide (25 μg/mL) and 5 nM /C0 for 18 h at room temperature, followed by 3 h incubation at 37°C. The stabilized HLA-A2 molecules on the cell surface were detected using FITC-conjugated anti-HLA-A2 antibody (Pharmingen). Labeled cells were analyzed by flow cytometry using a FACScan (Becton Dickinson). PDT treatment. The study was conducted in accordance with the Declaration of Helsinki. All patients were provided with written, informed consent. The Roswell Park Cancer Institute (RPCI) Institutional Review Board approved the study. Photosensitizers were applied prior to light application. Depending on the depth of the tumors, the physician determined whether patients were treated with 5-aminolevulinic acid (ALA)-PDT or Porfimer sodium-PDT. ALA was applied as a 20% moisture cream containing 240 mg of crystalline ALA mixed with 960 mg of liquid moisturel. The cream was applied in a thin layer over the lesion with about 0.5- to 1.0-cm border of normal skin 4 to 24 h before light application. The area was then covered with occlusive dressing to shield it from light and to keep the area moist until light application. Topically applied ALA was used primarily for thinner lesions as it has been shown to be less effective on thicker lesions due to lower permeability into the stratum corneum (26). Porfimer sodium (1 mg/kg) in D5W was infused into patients i.v., which allows for treatment of deeper lesions (27) but results in significant skin phototoxicity. Therefore, Porfimer sodium–treated patients were instructed to avoid direct sunlight as well as strong indoor lighting for up to 6 wk postinfusion. Light was applied directly on the lesions (both ALA and Porfimer sodium treatments) at a fluence of 100 to 260 J/cm2 at a fluence rate of 150 mW/cm2 4 h post-ALA application or 48 h post–Porfimer sodium infusion. Lesions from 2 to 7 cm in diameter were exposed to 630-nm light derived from a Spectra Physics Model 171 argon laser pumping a Spectra Physics tunable dye laser (Model 375). Light was delivered to the sites using 400-nm or 600-mm diameter quartz fibers fitted with microlenses to give spots of light with uniform intensities. A filtered tungsten-halogen lamp (590–700 nm) that allows adjustment of the diameter of the illumination field (DUSA Pharmaceuticals, Inc.) was used for larger fields with up to 16-cm diameter. Clinical follow-up was done at 3 and 6 mo post-PDT and then on an average of every 6 mo. Complete clinical responses were determined at ≥6 mo.

Blood collection and lymphocyte isolation. Blood from basal cell carcinoma patients was drawn in the Dermatology clinic or Laboratory Medicine Department at RPCI 1 to 2 d before and 7 to 14 d post-PDT treatment. Lymphocytes were isolated from peripheral blood mononuclear cells (PBMC) by centrifugation over a Ficoll gradient (Ficoll-Paque PLUS, Amersham Biosciences). Isolated lymphocyte samples were frozen at −70°C in 80% human serum albumin, 10% AIM V medium, and 10% DMSO until use. At the time of the assay, lymphocytes were thawed, washed twice in PBS, counted, and resuspended in complete medium (RPMI 1640 containing 10% fetal bovine serum). Determination of HLA-A2 expression. Patient lymphocytes (10⁶ cells) were incubated with a FITC-conjugated anti-HLA-A2 monoclonal antibody (Pharmingen). Samples were then analyzed by flow cytometry on a Becton Dickinson FACScan. Mean fluorescence intensity was compared with binding of cells to an isotype control antibody. ELISpot assay for IFN-γ. ELISpot plates (96 wells; Millipore) were coated with antihuman IFN-γ capture antibody (mAb1-D1K; Mabtech) at a concentration of 0.5 μg/mL in PBS overnight at 4°C. The wells were then washed with PBS and blocked with RPMI 1640 containing 10% fetal bovine serum for at least 2 h in a 37°C, 5% CO₂ incubator. T2 target cells were pulsed with HP peptide (25 μg/mL) or an irrelevant peptide. The HLA-A2–binding peptide, gag57-65, from HIV p17 (28), was used as an irrelevant peptide control. The media were discarded off the blocked ELISpot plates and patient lymphocytes (1 × 10⁶) were added to the wells in triplicate. The pulsed T2 (5 × 10⁵) cells were added to appropriate wells containing lymphocytes. The plates were incubated in a 37°C, 5% CO₂ incubator for 18 to 20 h. Plates were then washed several times in PBS containing 0.5% tween-20 (PBS-Tween). Biotin-conjugated anti-IFN-γ (mAb 7-B6-1; Mabtech) was added to each well and plate at 2 μg/mL and the plates were placed back in the incubator for 2 h. Plates were washed several times with PBS-Tween. Streptavidin-horseradish peroxidase (BD Pharmingen) was added to each well at a 1:1,000 dilution. Plates were incubated at room temperature for 1 h. IFN-γ spots were developed using AEC staining kit (AEC-101; Sigma). AEC solution (100 μL) was added to each well and incubated for 5 to 15 min. The plates were washed and dried in the dark. The number of spot-forming units in each well was enumerated by computer-assisted image analysis using the Zeiss ELISPOT reader system equipped with v.4.1.56 software (ZEISS) by the Roswell Park Cancer Center Immunomonitoring Core facility.

Translational Relevance
This study examines the ability of local photodynamic therapy (PDT) to enhance immune recognition of tumor cells following treatment of basal cell carcinoma lesions. Numerous preclinical studies have suggested that enhancement of antitumor immunity plays a critical role in the long-term cure rate mediated by PDT. With the exception of single-case and anecdotal reports, this is the first study to examine the effect of PDT on the immune response in a clinical setting. The findings support the hypothesis that PDT is able to enhance antitumor immunity and suggest that enhancement of antitumor immunity is inversely correlated with the extent of the surface area treated and light dose. These results support recent findings that low fluence rates and lower light doses can lead to more effective PDT and suggest that modifications of the current clinical practice of high light dose and fluence rates may result in the control of distant disease.

Patients and Methods
Identification of HLA-A2–binding Hip1 peptides. The Bioinformatics and Molecular Analysis Section (BIMAS) of the NIH (Bethesda, MD) was used to identify Hip1 peptides with HLA-A2–binding potential. BIMAS calculates a “theoretical” binding stability matrix based on the rate of dissociation from β2-microglobulin (β2M), which was developed by Parker et al. (24), to predict potential HLA-A2–binding peptides. Potential HLA-A2–binding Hip1 peptides were synthesized at the University of Georgia Molecular Genetics Instrumentation facility. Stock solutions of peptides were made in 100% DMSO and stored at −20°C until use. Working concentrations (500 μg/mL) of peptides were made in RPMI-1640. Peptide binding to HLA-A2 was confirmed using a modification of the T2 stabilization assay (25). Briefly 10⁶ T2 cells were incubated in RPMI-1640 in the presence of peptide (25 μg/mL) and 5 nM /C0 for 18 h at room temperature, followed by 3 h incubation at 37°C. The stabilized HLA-A2 molecules on the cell surface were detected using FITC-conjugated anti-HLA-A2 antibody (Pharmingen). Labeled cells were analyzed by flow cytometry using a FACScan (Becton Dickinson).

PDT treatment. The study was conducted in accordance with the Declaration of Helsinki. All patients were provided with written, informed consent. The Roswell Park Cancer Institute (RPCI) Investigational Review Board approved the study. Photosensitizers were applied prior to light application. Depending on the depth of the tumors, the physician determined whether patients were treated with 5-aminolevulinic acid (ALA)-PDT or Porfimer sodium-PDT. ALA was applied as

http://bimas.dcrct.nih.gov/
**Statistical analysis.** To describe the observed variability in the data and test for variables related to immune response, a series of mixed linear models were fit to the data. The dependent variable for all considered models was immune response following treatment, repeated measures of which were taken for each patient. Independent variables included the corresponding pretreatment value and random subject and day effects. The following variables were also included in the model one at a time: age, sex, diagnosis, drug, light dose, number of lesions treated, number of prior treatments, total area treated, and clinical response rates. Standard diagnostic plots were used to assess model fit and all statistical tests were carried out using SAS version 9.1.3 statistical software, and done at the 0.05 level of significance.

**Results**

**Identification of HLA-A2–binding peptides in Hip1.** Several preclinical studies have shown that PDT-enhanced antitumor immunity is dependent upon the presence of CD8+ T lymphocytes (reviewed in ref. 14). Tumor-specific CD8+ T cells respond to peptide epitopes bound to MHC I molecules. In order to determine whether PDT of basal cell carcinoma lesions enhanced immune recognition of Hip1, the basal cell carcinoma TAA, it was first necessary to identify MHC class I (MHC-I) binding peptides present in Hip1. HLA-A2 is the most common MHC-I allele in North America and its peptide binding motif is well characterized. Therefore, analysis of the full-length amino acid sequence of Hip1 was done using the BIMAS software program to predict Hip1 peptides capable of binding HLA-A2. BIMAS is a matrix-based algorithm that predicts peptide binding based on experimentally measured β2-microglobulin dissociation rates (24). Analysis revealed several peptides that could theoretically bind strongly to HLA-A2. The four peptides identified to have a high potential to bind HLA-A2 according to their dissociation constants were K105 (KMLSFKLLL), F243 (FILEKEGYV), I368 (ILGDGMITIL), and F518 (FLTLQQSPV; Table 1).

The T2 stabilization assay was used to confirm peptide binding to HLA-A2 (25). T2 cells are TAP1/2 deficient; TAP1/2 molecules are needed for the loading of endogenous peptides onto nascent MHC-I molecules, which is required for protein stability and cell surface expression. T2 cells do not stably express cell surface HLA-A2 molecules; however, the addition of exogenous peptides that bind to HLA-A2 results in the stabilization of HLA-A2 and cell surface expression. Figure 1 shows that peptides K105, F243, and F518 stabilize cell surface expression of HLA-A2 on T2 cells, with K105 showing the best binding ability. Peptide I368 did not exhibit HLA-A2 binding, which was surprising. However, the BIMAS algorithm depends on the assumption that each amino acid in the peptide contributes independently to binding and there are known cases where the combination of amino acids in the peptide do not behave as predicted, which is likely a result of the individual coefficients of binding not being determined accurately enough or a lack of knowledge of unfavorable amino acid preferences (24, 29).

**PDT enhances lymphocyte recognition of Hip1 peptide K105.** To determine whether clinical PDT enhanced immune recognition of TAAs, blood was collected from patients with nodular or superficial basal cell carcinoma 1 to 2 days prior to and 7 to 14 days after PDT. Lymphocytes were isolated from HLA-A2+ patients tested for reactivity to Hip1. Reactivity was determined using ELISpot assays to measure IFN-γ production following incubation of patient lymphocytes with Hip1 peptide K105. A minimum of three independent tests were done on each pretreatment and posttreatment sample.

Samples were collected from 60 patients, 40% of which were HLA-A2+. Of the 24 patients that were HLA-A2+, post-PDT samples were collected from 21 patients and from 4 patients whose lesions were surgically removed. The patient profile (age, sex, and diagnosis), treatment parameters (photosensitizer and light dose used), and responses (clinical and immune reactivity) are shown in Table 2. The PDT treatment population consisted of 12 males and 9 females and included 7 patients with nodular basal cell carcinoma and 14 patients with superficial basal cell carcinoma. Porfimer sodium–PDT was used to treat three patients, one with superficial basal cell carcinoma and two with nodular basal cell carcinoma. ALA–PDT was used to treat 13 patients with superficial basal cell carcinoma and 5 patients with nodular basal cell carcinoma. There was no significant difference in clinical response rates between patients treated with Porfimer sodium–PDT and ALA–PDT (evaluations made ≥6 months after PDT). The clinical response rate following treatment of superficial basal cell carcinoma with PDT was 92% and varied between 100% response and 50% response. The average clinical response rate for nodular basal cell carcinoma was less than that for superficial basal cell carcinoma, with an average response rate of 60.2% that varied between a response of 90.9% and no response.

Of the 21 patients treated with PDT, 17 exhibited an increased response to Hip1 following treatment and 15 of the 17 responding patients exhibited a ≥2-fold increase in their response when the posttreatment mean was compared with the pretreatment mean (Fig. 2). Treatment with PDT resulted in significantly higher immune reactivity when compared with surgery ($P \leq 0.03$); only one of the four patients whose lesions were removed surgically had a response that was more than twice the pretreatment response. The reason for the increase in this patient is unknown as there was no apparent difference in tumor burden or removed lesion size among the four patients.

The effect of PDT on reactivity to Hip1 was greater in patients following treatment of superficial lesions as compared with that of patients with nodular lesions. All of the patients who had superficial lesions treated with PDT showed ≥2-fold increase in Hip1 reactivity. In contrast, only 57.1% (4 of 7) patients treated for nodular basal cell carcinoma exhibited an increased

| Table 1. Predicted binding of Hip1 peptides to HLA-A2 |
|---------------------------------|---------|--------|
| Peptide | Sequence | Position (a.a.) | Score* |
| K105 | KMLSFKLLL | 105-113 | 607 |
| F243 | FILEKEGYV | 243-251 | 477 |
| I368 | ILGDGMITIL | 368-376 | 342 |
| F518 | FLTLQQSPV | 518-526 | 320 |

*Estimated Kd based on the BIMAS software package, which predicts MHC restricted peptides based on the amino acid sequence of the protein of interest.
reactivity to Hip1 following treatment, and only 14.2% or 1 of 7 patients had ≥2-fold change in reactivity.

Patients treated with light doses above 200 J/cm² exhibited lower increases in immune reactivity to Hip1. Recognition of Hip1 following PDT was inversely affected by amount of area treated \((P < 0.03)\), which is shown graphically in Fig. 3.

Interestingly, in two cases, patients 4 and 13, we observed that lesions present at the time of treatment, but outside the treatment field, seemed to regress following PDT treatment. Unfortunately, this study was not designed to follow regression of lesions outside the treatment field and thus these findings are limited and anecdotal.

### Table 2. Patient characteristics, diagnosis, treatment conditions

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Abbreviations: PS, photosynthesizer; I, increased recognition of Hip peptide; D, decreased or no change in recognition of Hip peptide.

*% of treated lesions with complete clinical response.
Discussion

Although numerous preclinical studies have shown that PDT of murine tumors can result in an increase in antitumor immunity (reviewed in ref. 14), to our knowledge the current study is the first to show that PDT in a clinical setting can enhance immune cell recognition of TAAs. Here we show that both ALA-PDT and Porfimer sodium–PDT enhance the immune response, as measured by IFN-γ secretion, against the basal cell carcinoma–associated tumor antigen Hip1. Importantly, immune reactivity following treatment was significantly greater in patients treated with PDT as compared with those whose lesions were removed surgically.

Abdel-Hady et al. (30) first suggested the importance of antitumor immunity to clinical outcome. They showed that patients with vulval intraepithelial neoplasia who did not respond to ALA-PDT were more likely to have MHC-I–negative tumors than patients who responded to ALA-PDT. Responding patients also exhibited increased CD8+ T-cell infiltration into their tumors after treatment as compared with nonresponders. In addition, a study by Dragieva et al. (31) on the use of ALA-PDT to treat actinic keratoses and Bowen’s disease in immunosuppressed and immunocompetent patients showed that although both patient groups had similar initial response rates of >80%, immunosuppressed patients exhibited persistence of disease or appearance of new lesions that was greater than that of immunocompetent patients. Finally, a recent case report showing that treatment of angiosarcoma with lower light dose and fluence rate resulted in remission of neighboring and distant untreated lesions (34), suggests that induction of antitumor immunity occurs at lower light dose and fluence.
rates. These findings are corroborated by the current results showing that treatment of basal cell carcinoma lesions with lower light doses resulted in greater enhancement of immune recognition of Hip1. The current study also supports subsequent study into the effect of PDT on basal cell carcinoma lesions present outside the treatment field as two of the patients showed regression of untreated lesions following PDT.

Both ALA and Porfimer sodium photosensitizers were used in this study. These photosensitizers are similar in their mechanism of tumor eradication, which occurs directly via tumor cell death due to singlet oxygen release and indirectly via vasculature disruption and inflammation (3). ALA-PDT is thought to have less vascular effects than Porfimer sodium–PDT (36), but the extent to which that influences tumor response is unclear. Both treatments were able to stimulate increased recognition of Hip 1 and no significant difference was observed between the responses of patients treated with ALA-PDT versus Porfimer sodium–PDT. In addition, there was no significant increase in either clinical response rates or Hip 1 recognition in the nodular basal cell carcinoma patients treated with Porfimer sodium–PDT as compared with those treated with ALA-PDT; the analysis is limited, however, by the low patient number.

Certain PDT regimens have been shown to systemically suppress immune reactivity in preclinical models (reviewed in refs. 37, 38). The switch from immune-enhancing to immune-suppressing effects of PDT seems to be linked to the area of skin treated; whole-body light irradiation in combination with photosensitizer resulted in immune suppression and reduction in autoimmunity in several model systems (37, 38). Our results show that enhancement of antitumor immunity is inversely related to the area treated, which may indicate that treatment of large surface areas leads to immune suppression rather than immune stimulation, although further study is required to confirm this finding.

The ability of PDT to enhance antitumor immunity suggests that this treatment modality may be used in an adjuvant setting with treatments that have either no or a negative effect on the patient immune response, such as surgery. Friedberg et al. (39) report increased survival for patients with non–small cell lung cancer with pleural spread who receive surgery and PDT when compared with patients receiving surgery alone. Pleural PDT is accompanied by an increase in inflammation (13), which has been linked to enhanced antitumor immunity following PDT (14, 33).

A number of studies have suggested that the enhancement of antitumor immunity following PDT is due to the release of immunogenic peptides and danger signals from dead/dying tumor cells (14, 15, 32), which leads to the activation of dendritic cells and increased stimulation of tumor-specific T cells. Dendritic cell activation and increased T cell stimulatory capacity can also be achieved by application of toll-like receptor agonists, such as imiquimod (40). Imiquimod has proven to be efficacious in the treatment of superficial basal cell carcinoma (41), and a recent study suggests that local application of imiquimod and photoimmunotherapy to advanced cutaneous melanoma lesions can result in systemic control of disease (42). Our study further supports the use of PDT as a means to enhance antitumor immunity in either a stand-alone or adjuvant setting.

In summary, we have shown that treatment of basal cell carcinoma with either Porfimer sodium–PDT or ALA-PDT results in an enhancement of the ability of immune cells to recognize and respond to the tumor-associated antigen Hip1. This is the first study to directly examine the ability of PDT to enhance antitumor immunity in a clinical setting. The findings provide rationale for further mechanistic studies and for the development of PDT regimens to be used in concert with other cancer therapy modalities to enhance long-term survival and the control of distant disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Enhanced Systemic Immune Reactivity to a Basal Cell Carcinoma Associated Antigen Following Photodynamic Therapy

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