Immunosuppressive Effects of Silicon Phthalocyanine Photodynamic Therapy

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ABSTRACT

The purpose of this study was to determine if silicon phthalocyanine 4 (Pc 4), a second-generation photosensitizer being evaluated for the photodynamic therapy (PDT) of solid tumors, was immunosuppressive. Mice treated with Pc 4 PDT 3 days before dinitrofluorobenzene sensitization showed significant suppression of their cell-mediated immune response when compared to mice that were not exposed to PDT. The response was dose dependent, required both Pc 4 and light and occurred at a skin site remote from that exposed to the laser. The immunosuppression could not be reversed by in vivo pretreatment of mice with antibodies to tumor necrosis factor-alpha or interleukin-10. These results provide evidence that induction of cell-mediated immunity is suppressed after Pc 4 PDT. Strategies that prevent PDT-mediated immunosuppression may therefore enhance the efficacy of this therapeutic modality.

INTRODUCTION

Photodynamic therapy (PDT) shows promise as an effective form of therapy for malignant tumors in a wide range of organ systems (1–7). Its efficacy has been demonstrated in the palliative treatment of esophageal cancer (1,6,8–11), early- and late-stage lung carcinomas (1,6,12) and in bladder tumors (1,5,6,13). Additional clinical studies attest to its value in the management of head and neck tumors (1,14–18), nonmelanoma skin cancer (1,6,19,20), Kaposi’s sarcoma (1,21), cutaneous breast metastases (1,22), ovarian carcinoma (1), malignant glioma (1,23) and a variety of other tumors (1). Photodynamic therapy involves systemic administration of a photosensitizing agent that localizes selectively within tumors (1–4,6,7). Alone the compound is inactive. However, when it is activated by exposing the tumor to high intensity visible light, usually from a laser, singlet oxygen is generated (1,3,4,24). These highly reactive molecules are damaging to cell membranes. This causes apoptosis of tumor cells and eventuates in tumor destruction (1,25,26).

The only photosensitizer currently approved for clinical use by the Food and Drug Administration and other regulatory agencies is Photofrin, a complex mixture of porphyrin oligomers (1). Although effective, Photofrin is activated by wavelengths of light (625 nm) that have a rather superficial depth of penetration within tissues that limits the efficacy of this treatment modality. Photofrin is also associated with prolonged cutaneous photosensitivity that may persist for weeks to months after it has been administered. Studies to evaluate its influence on the immune system have shown that, in animal models, it suppresses contact hypersensitivity to dinitrofluorobenzene (DNFB) (27–29).

A number of second-generation photosensitizers that either have improved efficacy or reduced side effects compared to Photofrin are currently being evaluated in PDT protocols (1,3). Studies from our group have focused on the silicon phthalocyanines (Pc) (30–32). These agents have a large macrocycle ring with silicon chelated in the center. Silicon Pc 4 has a single aminoisooxy ligand associated with it. In contrast to Photofrin, Pc 4 can be synthesized as a pure compound. Also, it is activated by 675 nm light that penetrates more deeply into tissues. In previous studies we have shown that PDT with Pc 4 is at least as effective as Photofrin at causing regression of RIF-1 tumors in C3H/HeN mice and causes little, if any, photosensitivity using the doses employed for PDT.

The purpose of this study was to evaluate the potential of Pc 4 PDT to cause immunosuppression because this biological effect has been described for other PDT photosensitizers.

MATERIALS AND METHODS

Mice. Adult female, birth date-matched C3H/HeN mice, 8–12 weeks of age, were supplied by Charles River Breeding Laboratories (Kentington, NY). Institutional guidelines regarding animal experimentation were followed. Mice weighed 18–25 g. Each panel of mice consisted of four or five mice.
Chemicals. The silicon Pc, Pc 4, was employed for these studies. Phthalocyanine 4 was obtained from the National Cancer Institute. Phthalocyanine 4 was dissolved in taxol vehicle for administration to animals. 2,4-Dinitro-1-fluorobenzene (DNFB) was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in an acetone : olive oil solution (4:1).

Photodynamic therapy with Pc 4 or with Photofrin. Mice were given intravenous injections of 0.25-0.6 mg/kg Pc 4 as described in the Results section. The volume of Pc 4 was adjusted to deliver 100 μl for every 10 g of body weight. Twenty-four hours later, the dorsal skin was exposed to 135 J/cm² 675 nm radiation from an argon-pumped (Coherent, Innova 100) dye laser (Coherent, 599). Hair was removed from the irradiation site by depilation at least 48 h prior to injection with the photosensitizing agent. Mice were anesthetized with an intraperitoneal injection of 0.2 ml of 1:1 ketamine : xylazine prior to irradiation. Then, 675 nm light was delivered using a 1 cm-diameter spot size aimed at the depilated aspect of the back of each mouse. A 1 cm-diameter spot size was chosen because that is the size that has been employed to treat murine tumors in our other studies (30,33). Ears were not within the field of light administration. After treatment with light, the mice were kept in a dark environment and were shielded from further light exposure.

Photofrin® was suspended in 5% dextrose and injected intravenously at a dose of 10 mg/kg. A dose of 0.05 ml was administered, for a total dose of 0.2 mg. This dose has been previously determined to be efficacious for the PDT of RIF-1 tumors (34). Photofrin was obtained from QLT Phototherapeutics, Inc. (Vancouver, BC, Canada). Twenty-four hours later, the animals were treated with light from an argon-pumped dye laser at a fluence of 145 J/cm². This fluence was chosen in order to deliver the same number of photons as that which was delivered when Pc 4 was employed as the photosensitizer.

Assessment of cell-mediated immunity. Cell-mediated immune function following Pc 4 PDT was assessed with DNFB contact hypersensitivity. Induction and elicitation of DNFB contact hypersensitivity was performed, modifying the methods described previously (35). Unless otherwise stated, mice were sensitized to DNFB on days 3 and 4 after exposure to light from the laser by applying 25 μl of 0.5% DNFB in an olive oil : acetone solution to the shaved abdomen. Four days after the second treatment with DNFB, one ear was challenged by topical administration of 20 μl of 0.2% DNFB to both sides of the ear. Measurements of ear thickness were taken prior to challenge, and 24 and 48 h after challenge using a dial-thickness gauge micrometer. Positive control panels were sensitized and challenged with DNFB but were not given Pc 4 or light prior to sensitization. Negative control panels did not receive Pc 4 PDT or DNFB sensitization but were challenged with DNFB on the ear. The difference in ear thickness when the pre- and post-DNFB challenge measurements were compared was used to quantify the development of cell-mediated immunity. Suppression of the immune response was calculated using the following formula:

% suppression = [ear swelling (positive control) - ear swelling (experimental)] + [ear swelling (positive control) - ear swelling (negative control)] × 100.

In vivo treatment with cytokine antibodies. Mice were treated with rabbit anti-mouse tumor necrosis factor-alpha (TNF-α) antibodies (20 μl with a neutralizing capacity of 2000 units of TNF-α; Genzyme, Boston, MA) 3 days prior to illumination of animals using previously described methods that inhibited Pc 4 PDT phototoxicity (33). Twenty-five microliters of anti-mouse interleukin (IL) -10 antibody (Genzyme, Boston, MA) was injected intraperitoneally 3 days prior to exposure of skin to light (36,37). In preliminary experiments, the antibodies maintained their in vivo neutralizing effects for at least 6 days.

RESULTS

Initial studies were performed to evaluate whether or not suppression of cell-mediated immunity was a consequence of Pc 4 PDT. Panels of mice were injected intravenously with 0.6 mg/kg of Pc 4. Twenty-four hours later, a 1 cm spot of depilated skin on the back was exposed to 135 J/cm² 675 nm light. This treatment regimen resulted in blanching of the skin that by 5 days evolved into an eschar, comparable to that which is observed when tumors are treated with Pc 4 PDT. Three days after that, attempts were made to immunize mice to DNFB by applying 25 μl of 0.5% DNFB daily for 2 consecutive days to the abdomen. This site was remote from the area of light exposure (i.e. back skin). Four days later, the ears of animals were challenged, and the increment in ear thickness was used to quantify the magnitude of the immune response. As shown in Fig. 1, the Pc 4 PDT-treated panel showed 46% immunosuppression 24 h after challenge and 30% at 48 h.

For comparison purposes, separate panels of mice were given Photofrin PDT. Animals were then treated in an identical manner to those that had been treated with Pc 4 PDT and contact sensitized to DNFB with the exception that the mice were given an intravenous injection of 0.2 mg Photofrin at a dose of 10 mg/kg, followed 24 h later by exposure of a 1 cm area of skin to 145 J/cm² 625 nm light. As shown in Fig. 2, mice treated with Photofrin PDT exhibited 49% suppression of the DNFB contact hypersensitivity response, which was comparable to that was observed with Pc 4 PDT (Fig. 1). To exclude the possibility that Pc 4 alone or light alone was responsible for the immunosuppressive effects of this therapeutic modality, panels of mice were treated with Pc 4 alone, vehicle and light or Pc 4 plus light. They were then immunized to DNFB according to the protocol described above. Comparison was made to positive controls that were sensitized to DNFB but were not treated with Pc 4 or light and to negative controls that were neither treated with Pc 4 PDT nor immunized to DNFB. The results are shown in Fig. 3. Although significant suppression was observed in animals treated with Pc 4 and light, administration of Pc 4 alone or light alone resulted in little or no suppression of DNFB contact hypersensitivity. These results indicate that both Pc 4 and light are required for suppression of the immune response and that neither alone is sufficient.

Figure 1. Phthalocyanine 4 PDT is immunosuppressive. Animals were treated with Pc 4 PDT to normal skin on the back. Their immune response was then assessed by sensitizing animals with the contact allergen DNFB as described in the Materials and Methods.
Dose–response studies with Pc 4 were conducted in which panels of mice were treated with 0.25, 0.375 or 0.5 mg/kg Pc 4 and then exposed to 135 J/cm² 675 nm light. There was a direct correlation between the dose of Pc 4 used for PDT and the magnitude of the immunosuppression (Fig. 4).

The effect of timing on the development of immunosuppression was also investigated. To do this, panels of mice were treated with Pc 4 and light. One group was immunized to DNFB immediately after PDT. The other panel was immunized 3 days after PDT. Comparison was made to positive control mice that were sensitized to DNFB but were not treated with Pc 4 or light. In animals immunized to DNFB immediately after irradiation, no suppression of the cell-mediated immunity was observed. In contrast, animals treated with Pc 4 PDT 3 days prior to DNFB immunization exhibited 60% suppression of cell-mediated immunity (Fig. 5). These results indicate that the immunosuppression produced by Pc 4 PDT is not immediate but requires time to develop.

The fact that animals received Pc 4 PDT on the back but were tested for immune responsiveness on an area of skin remote from the irradiated site and that Pc 4 PDT-induced immunosuppression took 3 days to develop suggested to us that an immunosuppressive soluble mediator is elaborated following PDT. Observations with respect to UV radiation-induced immunosuppression have indicated that IL-10 and TNF-α are likely candidates as mediators for that form of immunosuppression (36). To investigate the role of TNF-α and IL-10 in Pc 4 PDT-induced immunosuppression, neutralizing antibodies to these cytokines were administered in vivo to panels of mice 3 days prior to light from a laser. Three days after that, attempts were made to immunize mice with DNFB. Antibodies to TNF-α provided modest reversal of immunosuppression that was not statistically significant (Fig. 6). Antibodies to IL-10 had no effect on immunosuppression (Fig. 7). Moreover, administration of antibodies less than 3 days prior to PDT not only failed to reverse the immunosuppression but also interfered with elicitation of DNFB contact hypersensitivity response (data not shown).

**DISCUSSION**

Previous studies, in both animal and human models, have shown that UV radiation can cause suppression of cell-mediated immune reactions (35,38). Similar results have been obtained with selected photosensitizers and light (26–29,39,40). The results presented here provide evidence that inhibition of cell-mediated immunity is a consequence of Pc
4 PDT. In this regard, Pc 4 PDT is similar to other modalities used to treat solid tumors. Chemotherapy, radiation therapy, and even surgery, which cause large-scale tissue destruction, are all associated with immunosuppression (41,42).

The animals were treated in this study in an identical manner to those treated for tumor regression with Pc 4 PDT (1 cm spot size, 0.4 mg/kg PC 4, 135 J/cm²). The only exception is that non-tumor-bearing mice were employed for these experiments in order to exclude the potential immunosuppressive effects of the tumor itself. With other photosensitizers, PDT-induced immunosuppression is known to depend on the extent of the body surface area being illuminated. The fact that a 1 cm spot size was employed, which is a much larger proportion of the total body area on the mouse than it is on the human, raises the question as to whether the findings in this study can be extrapolated to humans. Because there have been no studies examining whether PDT suppresses the induction of cell-mediated immune responses in humans, it is impossible to answer the question directly. However, it is important to emphasize that in humans, it is not unusual to treat up to 30 or more skin tumors at one time with PDT, which is a significant proportion of the total body surface area. Furthermore, the extent of UVB-induced immunosuppression in mice also depends on the total body surface area being irradiated (43). However, when humans have been subjected to a similar UVB protocol, immunosuppression can be achieved by illuminating as small as a 2 cm area of skin (44,45). This suggests that humans may be much more susceptible to at least some forms of immunosuppression than mice.

The observation that PDT is immunosuppressive may open new opportunities for optimizing protocols for its use in clinical situations. For example, it is conceivable that PDT-induced tumor regression may occur at the expense of the generation of an effective antitumor response. Thus, procedures that limit PDT-induced immunosuppression but do not affect PDT-induced regression of tumors may prove to be superior to PDT alone in promoting long-term tumor responses. It is well known that tumor-specific T cells can eliminate small foci of malignant cells, and this is likely to be the way in which tumor cells that have escaped the direct cytotoxicity and vascular effects of PDT are removed.

Strong evidence that PDT, in fact, does lead to the induction of tumor-specific immunity comes from studies in which EMT-6 mammary carcinoma cell line was transplanted to BALB/c mice (46-48). Photodynamic therapy caused marked tumor regression and resulted in long-term tumor cures. In contrast, when the same tumors were transplanted to immunodeficient nude or severe combined immune-deficient (scid) mice that were then subjected to Photofrin PDT, tumor regression could be achieved, but there were no long-term cures. When cells from spleen or bone marrow of immunocompetent BALB/c mice were transferred to scid mice, recurrence was delayed, providing further evidence that the immune system contributes in an important way to the efficacy of PDT. The finding that treatment with the adjuvant serum vitamin D₃-binding protein-derived macrophage-activating factor (DBPMAF) enhanced the curative effect of PDT against the SCCVII tumor provides additional support for a role of the immune system in PDT-induced tumor regression and cure (47).

The mechanism by which Pc 4 PDT causes immunosuppression is open to speculation. We were surprised to find that unlike UVB-induced immunosuppression, Pc 4 PDT-mediated suppression was not inhibited by TNF-α or IL-10. In other studies, others and we have found that PDT in general and Pc 4 PDT in particular is a potent stimulus for TNF-α production (33). This adds to the differences between PDT- and UVB-induced immunosuppression. Ultraviolet-B immunosuppression of this form of contact hypersensitivity is mediated by DNA damage (49). Although the initial effects that result in PDT-induced immunosuppression have not been investigated, virtually all of the other biological effects of PDT are caused by the generation of reactive oxygen intermediates (1-4,6). Another difference between PDT- and UVB-induced immunosuppression is the cell type that is responsible for the immunosuppression. Ultraviolet-B-induced immunosuppression is mediated by regulatory T cells (28,35), whereas PDT-induced immunosuppression is thought to result from the activity of macrophages that regulate the immune response (50).

The fact that Pc 4 is immunosuppressive opens a whole new area in which this novel therapeutic agent might be
useful. It may be possible to develop protocols in which Pc 4 PDT is employed as an immunosuppressive modality in situations in which dampening of the immune response might be useful.

In summary, our results provide evidence that following Pc 4 PDT there is inhibition of cell-mediated immune responses and that the mechanism by which immunosuppression is achieved differs from that of UVB-induced immunosuppression. Strategies that prevent PDT-induced immunosuppression may therefore enhance the efficacy of this new therapeutic modality.

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REFERENCES


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