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Antitumor immunity induced by photodynamic therapy with aluminum disulfonated phthalocyanines and laser light

Gianfranco Canti,[1] Donatella Lattuada,[1] Angelo Nicolin,[1] Paola Taroni,[2] Gianluca Valentini[2] and Rinaldo Cubeddu[2]

[1]Department of Pharmacology, School of Medicine, University of Milano and [2] CEQSE, CNR, Via Vanvitelli, 32, 20129 Milano, Italy. Tel: (+39) 2 70146224; Fax: (+39) 2 70146381.

Photodynamic therapy (PDT) is systemic administration of tumor localizing photosensitizers and subsequent irradiation with light of the appropriate wavelength. The combination of drug uptake in malignant tissues and selective delivery of laser-generated light provides effective therapy, with efficient tumor cytotoxicity and minimal normal tissue damage. There have been few studies of the effects of photoactivated photosensitizers on the host immune response. Since immunity is important in the control of tumor growth and spread, we have examined, in our laboratory, the effects of photoactivated phthalocyanines on the antitumor immune response. Immunosuppressed and normal mice bearing the MS-2 fibrosarcoma treated with 5 mg/kg of aluminum disulfonated phthalocyanine (AlS_2Pc) and then the tumor mass exposed to laser light (100 mW/cm^2 × 10 min) or treated with surgical excision of the tumor survived indefinitely, with no difference between the different groups. The survivors, tumor-free 100 days after the treatment modalities described above, were rechallenged with the parental MS-2. Some groups of surviving animals were immunosuppressed with cyclophosphamide before the injection of the tumor. Resistance to rechallenge was evidenced only in normal surviving animals cured by PDT, while the immunodepressed surviving animals and animals cured by surgery died of tumor. Finally, mice, cured by PDT and tumor-free, rechallenged with L1210 and P388 murine leukemias did not survive. These results suggest that a potential and specific 'antitumor immunity' is induced by PDT with photoactivated AlS_2Pc.

Key words: Antitumor immunity, aluminum disulfonated phthalocyanines (AlS_2Pc), laser light, photodynamic therapy (PDT).

Introduction

A major goal of cancer treatment is selective destruction of malignant cells with preservation of normal tissues and functions. Photodynamic therapy (PDT) destroys malignant tumors through preferential uptake by neoplastic cells of photosensitizing compounds, which are then activated by suitable light exposure.[1,2] Activated photosensitizers interact with molecular oxygen to produce singlet oxygen that destroys neoplastic cells with minimal normal tissue damage.[3] *In vitro* and *in vivo* studies indicate that PDT kills cells both directly and indirectly as a result of reduced blood flow in and towards the tumor.[4]

PDT has been demonstrated to be effective in eradicating transplanted tumors in experimental animals.[1] PDT utilizing the hematoporphyrin derivative (Hpd) has been used clinically for palliation of obstructive lesions of the esophagus[5] and the tracheobronchial tree,[6] for treatment of bladder tumors[7] and for local control of various tumors on the skin surface.[8]

Toxicological and immunopharmacological properties of the photosensitizer most used in clinical oncology, Hpd, with and without exposure to light, have been investigated by several groups.[9,10] In our laboratory we have observed various effects of Hpd on different immune system functions.[11,12] We have demonstrated that Hpd induced spleen and bone marrow hyperplasia and increased the blastogenic responses of T and B lymphocytes.[13] However, other investigators have shown that the combination of Hpd and light exposure abrogated the ability of peripheral blood mononuclear cells to act as stimulator cells in a mixed lymphocyte reaction[14] and induced immunosuppression that is adoptively transferred by macrophages.[15] In addition, Hpd decreased dinitrofluorobenzene-induced hypersensitivity.[16] Recently it has been published that a complete, though transitory, suppression of natural killer (NK) cell activity can be achieved by treatment of mice with Hpd and laser light.[17] Thus, in the literature, the effects of Hpd-mediated PDT on the host immune defense mechanism are controversial and not definitive. In addition, Hpd has certain

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Correspondence to G Canti

disadvantages for use in PDT; it is a complex mixture of phorphyrins and absorbs red light poorly. This is a major disadvantage in PDT treatment, in which it is better to use red or near-infrared light (600–800 nm) for optimal tissue penetration.

Many laboratories are studying new photosensitizers, one of which is the aluminum disulfonated phthalocyanine (AlS_2Pc). It is water soluble, nontoxic and, unlike porphyrins, it strongly absorbs clinically useful red light.[18] Furthermore, AlS_2Pc has photosensitizing activity both *in vitro* and *in vivo*.[19]

However, as far as we can ascertain, few studies have been done on the interaction of phthalocyanine dyes with the antitumor immune mechanism.[20] In preliminary experiments, in our laboratory, we observed that photoactivated AlS_2Pc, like Hpd, induces spleen and bone marrow hyperplasia and increases the blastogenic response of T and B lymphocytes.[21]

Since immunity is important in the control of tumor growth and spread, we have evaluated, in the present study, the effects of PDT with photoactivated AlS_2Pc on antitumor immunity and compared PDT with surgical excision of a murine tumor.

Material and methods

Animals and tumors

Inbred BALB/c and hybrid DBA/2 × BALB/c male mice, 8–10 weeks old, obtained from Charles River (Calco, Italy) were used and are hereafter called BALB and CDF_1, respectively. MS-2 fibrosarcoma, originally induced by the Moloney murine sarcoma virus,[22] was maintained in the laboratory by weekly i.m. passage of tumor cell homogenate into the right hind leg of BALB mice. L1210 leukemia and P388 lymphoma, provided by National Cancer Institute, (Bethesda, MD), were transplanted weekly i.p. into CDF_1 mice.

In all the experiments, tumors were removed from mice under sterile conditions, a cell suspension was obtained by homogenization in a Potter, cells counted under optical light microscopy and injected intradermally (i.d., 10^6 cells/mouse). For the challenge inocula in surgically excised and PDT treated mice, the same cell preparations were used for all groups of mice.

Tumor surgical excision

Mice were anesthetized with phenobarbital i.p. A skin incision was made all around the neoplasia, as far as possible around the area of tumor growth. The skin with the tumor mass was cut with sterile scissors and wounds were closed by clamping with metal wound clips.

Chemicals

AlS_2Pc, kindly provided by Professor TG Truscott (Department of Chemistry, University of Keele, UK), was dissolved in 0.9% saline at a concentration of 5 mg/cm^3. Cyclophosphamide (Cy; Sigma) was dissolved in 0.9% saline at a concentration of 20 mg/cm^3.

Photodynamic therapy

Treatment was begun when the tumor mass measured approximately 1 cm in diameter. The animals were injected i.v. with 5 mg/kg of AlS_2Pc and irradiated with a single irradiation of laser light (100 mW/cm^2 × 10 min of exposure) 24 h later. The laser light was provided by a Kiton-red dye laser pumped by an argon laser. The dye was tuned at 670 nm. The laser output was coupled into an optical fiber (400 nm diameter) and the tumor lesion was illuminated with the fiber cut flat at the end. The laser power, at the end of the fiber, was measured by a thermopile (Orphyr 30A).

Results

The antitumor immunity induced by PDT was evaluated in normal and immunosuppressed mice bearing the poorly immunogenic MS-2 fibrosarcoma.[22] Immunosuppression of the animals was obtained by treatment with 200 mg/kg of Cy at day −1 before the transplantation of the tumor or at day + 5, 24 h before the injection of AlS_2Pc (Table 1). Six days after the tumor transplantation, normal and Cy immunosuppressed mice were injected with 5 mg/kg of AlS_2Pc and 24 h later the tumor masses were exposed to laser light (100 mW/cm^2 × 10 min of exposure). A 5 mg/kg dose of drug and the light dose of 100 mW/cm^2 × 10 min were adopted because of the optimal therapeutic protocol previously obtained.[23]

Large majorities of the animals (11/12, 10/12 and 10/12) injected i.d. with 10^6 MS-2 cells and treated with AlS_2Pc and laser light (PDT) survived indefinitely without any difference in survival between normal and immunosuppressed mice (Cy at day

Table 1. Treatment of mice bearing MS-2 fibrosarcoma by PDT or surgery

Treatment					MST (days)	D/T
day −1 drug	day 0 tumor	day +5 drug	day +6 dye	day +7 laser or surgery		
—	MS-2	—	—	—	35 (32–40)	7/7
Cy	MS-2	—	—	—	33 (29–35)	7/7
—	MS-2	Cy	AlS_2Pc	laser	— (44)	1/12
—	MS-2	—	AlS_2Pc	laser	— (35,66)	2/12
Cy	MS-2	—	AlS_2Pc	laser	— (40–65)	2/12
—	Ms-2	Cy	—	surgery	— (48)	1/12
—	Ms-2	—	—	surgery	— (50)	1/12
—	MS-2	—	—	surgery	—	1/12

CDF_1 mice challenged i.d. with 10^6 MS-2 fibrosarcoma cells. AlS_2Pc, 5 mg/kg, i.v.; Cy, 200 mg/kg, i.p.; laser, 100 mW/cm^2 × 10 min. D/T, dead animals/total; MST, median survival time.

−1 or day +5). The same results were obtained after surgical excision of the tumor masses. In fact, the majority of mice (11/12, 11/12 and 10/12) survived indefinitely after surgical excision, without any difference in survival between normal and immunosuppressed mice (Cy at day −1 or day + 5).

The normal and immunosuppressed control mice bearing MS-2 died without any difference in median survival time.

Post-surgical long-term survivors, tumor-free 100 days after surgical excision of the tumor masses, were rechallenged i.d. with 10^4 or 10^5 cells of the parental MS-2 tumor (Table 2). A challenge with 10^6 cells killed all the animals, with no difference in median survival time (MST) from the control group (unpublished observation). Two groups of the animals surviving from surgical excision (one with 10^4 challenge and one with 10^5 challenge) were immunosuppressed with Cy before the injection of the tumor. The animals in all groups died without any difference in MST between normal and immunosuppressed mice, suggesting that the surgical modality is not able to induce any antitumor immunity.

PDT treated long-term survivors (tumor-free 100 days after PDT) were rechallenged with 10^4 or 10^5 cells of the parental MS-2 tumor (Table 3), derived from the same cell preparation used for the rechallenge of the mice cured by surgery (Table 2). As in post-surgical mice, a challenge with 10^6 cells killed

Table 2. Resistance of animals, cured by surgery, to rechallenge with MS-2 tumors

Treatment		MST (days)	D/T
day −1 drug	day 0 tumor cells/mouse		
—[a]	10^4	54 (49–62)	6/6
Cy	10^4	57 (49–60)	6/6
—	10^4	58 (50–62)	6/6
—[a]	10^5	44 (43–50)	6/6
Cy	10^5	45 (40–51)	6/6
—	10^5	43 (38–47)	6/6

CDF_1 tumor free mice rechallenged i.d. with MS-2 fibrosarcoma. Cy, 200 mg/kg, i.p. D/T, dead animals/total; MST, median survival time.
[a]Control group = CDF_1 virgin mice challenged i.d. with MS-2 fibrosarcoma.

all the animals, with no difference in MST from the control groups (unpublished observation). As in the previous experiment, two groups of animals surviving after PDT (one with 10^4 challenge and one with 10^5 challenge) were immunosuppressed with Cy before the injection of the tumor. Strong resistance to both challenges was evident in the normal surviving animals while the immunosuppressed surviv-

Table 3. Resistance of animals, cured by PDT, to rechallenge with MS-2 tumor

Treatment		MST (days)	D/T
day −1 drug	day 0 tumor cells/mouse		
—[a]	10^4	52 (49–50)	6/6
+	10^4	50 (48–55)	6/6
—	10^4	— (80)	1/6
—[a]	10^5	42 (38–54)	6/6
+	10^5	43 (39–49)	6/6
—	10^5	— (76–83)	3/6

CDF_1 virgin mice rechallenged i.d. with MS-2 fibrosarcoma. Cy, 200 mg/kg, i.p. D/T, dead animals/total; MST, median survival time.
[a]Control group = CDF_1 virgin mice challenged i.d. with MS-2 tumor.

Table 4. Resistance of animals, cured by PDT, to a challenge with L1210 or P388 murine tumors

Tumor	Day 0	MST (days)	D/T
L1210[a]	10^4	12 (9–14)	3/3
L1210	10^4	12.5 (9.5–14)	3/3
P388[a]	10^4	12.5 (10–15)	3/3
P388	10^4	13 (10–16)	3/3

CDF_1 mice, tumor-free, challenged i.d. with L1210 and P388. D/T, dead animals/total; MST, median survival time.
[a]Control groups = CDF_1 virgin mice challenged i.d. with L1210 or P388.

ing mice died, without any difference in median survival time from the control groups. These results suggest that PDT with photoactivated AlS_2Pc had induced an 'antitumor immunity.'

Finally, the remaining animals surviving after PDT (Table 1) were rechallenged with tumors of different origin (leukemia L1210 and lymphoma P388). As is evident in Table 4, all the animals died, without any differences in MST, suggesting that PDT, induces a specific immune response, only against the parental tumor MS-2.

Discussion

PDT was effective in controlling malignant neoplasma in experimental animals,[1] and has been shown to be clinically useful in treating malignant neoplasma of the skin[8] and on mucosal surfaces of the tracheobronchial tree,[6] esophagus[5] and bladder.[7] The aim of PDT is to completely destroy the tumor; but this therapy often incompletely destroys the neoplastic mass or is followed by recurrence of the tumors. Cure requires total elimination of the cancer cells in the targeted tissues and also of the metastasized cancer cells. Therefore, it would be advantageous if the metastasized or undestroyed cancer cells could be eliminated immunologically rather than through chemotherapy. Surprisingly there have been few studies on the effects of photosensitizing dyes, with or without exposure to light, on host antitumor immune defense, and the results obtained are controversial and not definitive. Therefore, in the present study, we examined the effects of PDT with photoactivated AlS_2Pc, one of the active 'second generation' photosensitizers, on antitumor immunity. Normal and immunosuppressed mice bearing MS-2 fibrosarcoma and treated with PDT or by surgical excision of the tumor mass survived indefinitely (Table 1), as already reported in the literature.[23] When survivors 100 days after surgery were rechallenged with the parental tumor MS-2, they died from the tumor, suggesting that the surgery modality did not induce any antitumor immunity (Table 2). Instead, when the animals surviving 100 days after PDT with photoactivated AlS_2Pc were rechallenged with the parental tumor, they survived indefinitely, suggesting that PDT was able to induce a strong antitumor immunity. Two groups of these animals, cured by PDT, that had impaired immune responses, due to previous treatment with an immunosuppressive drug such as Cy, were not able to recognize and reject the parental tumor cells. In contrast, normal mice, cured by PDT, and not treated with Cy, were able to raise a remarkable 'antitumor immunity' against the parental tumor with its rejection (Table 3). We also observed that the PDT-induced antitumor immunity is restricted to the parental tumor, suggesting that the immune response is specific (Table 4). The time interval (100 days between surgery or PDT and challenge) was chosen because in previous experiments we observed that after this time interval we can be sure that the animals are cured and will be tumor-free, without any recurrence.

We have already published that Hpd and AlS_2Pc increased the blastogenic responses of T and B lymphocytes in normal,[13] irradiated[24] and tumor-bearing animals.[21] In one preliminary experiment, we also observed resistance to a rechallenge with MS-2 parental tumor by Hpd-mediated PDT-treated mice (unpublished observation). Other investigators have demonstrated that photoactivated Hpd activates murine macrophages *in vitro*[10] and these macrophages can be tumoricidal for cancer cells.[25] They suggested that damaged cancer cells can be efficiently phagocytized by the activated macrophages, leading to development of immunity against a cancer cell antigen. Since macrophages are antigen-presenting cells, activation of macrophages is a primary step in immunopotentiation. We have also shown that photoactivated AlS_2Pc increases the blastogenic response of lymphocytes[21] and it is known from the literature that lymphocytes are involved in the specific immune response against tumor antigen.[26] Therefore we suppose that both mechanisms, i.e. macrophage activation and increase in lymphocytes blastogenic response, may be involved in the photodynamic immunopotentiation.

In conclusion, the results of the present study could be of clinical interest since photodynamic immunopotentiation with photoactivated AlS_2Pc might improve the probability of curing cancer by PDT. We are currently carrying out additional studies with other photosensitizers and other murine tumors to confirm these results, and to identify the immune mechanism involved in this enhancement of the antitumor immune response.

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