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Combination of photodynamic therapy + immunotherapy + chemotherapy in murine leukiemia

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Photodynamic therapy (PDT) is a treatment for cancer based on the photosensitization of tumor cells by photosensitive drugs and their subsequent destruction on exposure to light of particular wavelength. The combination of drug uptake in malignant tissues and selective delivery of laser-generated light provides an effective therapy with efficient tumor citotoxicity and minimal normal tissue damage. Since immune response of the host is important in the control of tumor growth and spreading, PDT is able to increase the antitumor immunity. In our laboratory we examined the antitumor effect of combination of PDT,with photoactivated M-THPC (meta-tetrahydroxyphenylchlorin, FOSCAN, Temoporphirin), adoptive immunotherapy, with immune lymphocytes, and chemotherapy on advanced murine tumors. Mice bearing L1210 tumor were treated at day +4 with Navelbine (NVB 1mg/Kg), at day +5,+6 with PDT (0.3mg/Kg of mTHPC and 100mW/cm2 x 200" of exposure of laser light),and at day + 7 with immune lymphocytes(IL), collected from mice pretreated with PDT($2x10^7$ cells). The results show that the combination NVB + PDT + IL demonstrates a significant synergistic antitumor effect while the chemotherapy treatment with low dose of the drug is uneffective. The same positive results were obtained with the combination of Cisplatin (CDDP 0.5mg/Kg), PDT and IL, while the CDDP treatment alone is completely uneffective. In conclusion, these results suggest that it is possible to completely cure animals bearing advanced tumors, with a combined therapy, PDT + adoptive immunotherapy + low dose chemotherapy.

Key words: photodynamic therapy, combined therapy, immunotherapy, chemotherapy, L1210 murine leukemia.

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. Activated photosensitizers interact with molecular oxygen to produce singlet oxygen that destroys neoplastic cells with minimal normal tissue damage [1, 2].

The PDT-mediated antitumor effect is oxygen dependent and is the consequence of direct cytotoxicity and an antivascular effect, which impairs blood supply to the area [3]. The most widely studied PDT drugs both in experimental and clinical trials have been hematoporphyrin derivative and Photofrin (a complex mixture of monomeric and oligomeric porphyrins). PDT, utilizing the Photofrin (Ph), has been used clinically for palliation of obstructive lesions of the esophagus [4] and the tracheobronchial tree [5] , for treatment of bladder tumors [6] and for local control of various tumors on the skin surface [7]. Persistent cutaneous photosensitivity, known as a major side effect of systemic PDT, may lead to erythema, or blistering of light-exposed skin, if patients ignore the proper protection from sunlight [8]. This acute inflammatory reaction and the histological changes of PDT-treated tisssue with infiltrated lymphocytes, plasma cells and histiocytes, suggest a mayor immunological component in the response after photosensitization [9]. Photodynamically induced changes in the plasma membrane and membranes of cellular organelles, which represent the most abundant damage with a majority of photosensitizers used for PDT, can trigger events with far-reaching consequences. One process initiated at the membrane level involves signal trasduction pathways. These include enhanced expression of stress proteins and early response gene [10], activation of genes regulating the process of apoptotic cell death and possibly the up-regulation of some cytokine genes.

Due to their role in cell adhesion and antigen presentation, some of the PDT- induced stress proteins may participate in the development of inflammatory/immune response manifested by this therapy. A strong inflammatory reaction is a central event in the mechanism of PDT –mediated tumor destruction with the release of a wide variety of potent mediators like vasoactive substances, components of the complement cascades, cytokines (IL-6, IL-1β,IL-2,tumor necrosis factor-α and granulocyte colony-stimulating factor) growth factors and other immunoregulators [11, 12, 13]. Furthermore some photosensitizers, shown to stimulate the hematopoiesis in treated mice, may induce cytokine or growth factors independently of light treatment [14].There are indications that the tumoricidal activity of these activated inflammatory cells makes an essential contribution to the antitumor effect of PDT [15]. We have already shown that PDT is able to induce a strong antitumor immunity in tumor bearing mice and that the lymphocytes population can play an important role in the PDT modulation of immune response [16]. PDT induced antitumor immunity has similarities to the immune reaction induced by tumor inflammation caused by bacterial vaccines or some cytokines. Thus, although the PDT treatment is localized to the tumor site, its effect can have systemic attributes due to the induction of an immune reaction. PDT generated tumor sensitised lymphocytes can be recovered from distant lymphoid tissues (spleen, lymphonodes) at protracted times after light treatment. Therefore PDT can be successfully combined with various immunotherapy protocols for achieving substantial gains in long-term tumor controls.

With the discovery of second generation photosensitizers with a longer absorption wavelength(into the infrared spectrum) that permits better tissue penetration, there has been a renewed interest in PDT in oncology. M-THPC (meta-tetrahydroxyphenylchlorin, FOSCAN, Temoporphirin) is a second generation photosensitising agent used in PDT of tumors [17].

A logical way of reinforcing cancer therapy would be to consider the use of PDT in combination with other modalities like chemotherapeutic agents and adoptive immunotherapy to enhance effective regimens, and to permit some sparing of cytotoxic drugs so as to lessen their side effects.

Aim of this research is to study the effect of PDT with M-THPC (meta-tetrahydroxyphenylchlorin, FOSCAN, Temoporphirin), and laser light on lymphocytes in vivo and to utilize these activated immune lymphocytes for adoptive immunotherapy experiments, furthermore to analyse the antitumor effect of the combination therapy "PDT, immune lymphocytes and low dose of antiblastic chemotherapy" in mice bearing an aggressive malignant leukemia.

Materials and methods

*Animals and tumor.*All experiments were carried out in accordance with protocols approved by the local experimental animal welfare committee and conformed to national regulations for animal experimentation. Hybrid DBA/2 x BALB/c male mice, 8 – 10 weeks old, obtained from Charles River (Calco,Italy) were used and are hereafter called CDF1. Each group comprised six mice. L1210 murine leukemia was obtained from the Italian Tumor Institute, Milano, Italy and maintained by intraperitoneal (i.p.) injection of 10^6 cells/mouse in CDF1 male mice.

Chemicals. Cyclophosphamide (Cy) were supplied by the Italian Tumor Institute, Milano, Italy and dissolved in physiological solution.

Cisplatin (CDDP – Pfizer)was dissolved in Nacl,Hcl diluted 10% m/m with distilled water.

Navelbine (NVB – Pierre Fabre) was dissolved in physiological solution.

Foscan (mTHPC – Biolitec) was dissolved in Ethanol, anhydrous (40% w/w), propylene glycol.

*Laser source***.** Irradiation was applied with a continuous wave dye (DCM) laser pumped by an Argon laser and tuned at 670 nm. The laser output was coupled to a 400 μm plastic-glass optical fiber. The laser power was monitored at the fiber output.

Experimental procedure. L1210 ascitic tumor was drawn from the peritoneum of mice bearing the tumor and the cell suspension was counted under optical light microscopy. Tumor cells (10^6 cells/mouse) were injected intradermally (i.d.) to obtain a visible tumor mass that could be easily effectively irradiated by the laser light. Treatment started when the tumor mass measured approximately 0.5 cm in diameter (day 4). At days +4 the mice were treated with NVB (1 mg/kg) or CDDP (0.5 mg/kg). At day +5 the animals were injected intraperitoneally (i.p.) with 0.3mg/kg of Foscan and 24 hrs. later were irradiated with a single dose of light (100 mW/cm2 x 200" of exposure, energy density of 100J/cm2). At day +7 the mice were treated with immune lymphocytes (IL).

Winn assay. The in vivo antitumor activity of T cells was determined by the Winn tumor neutralization assay. Effector lymphocytes, collected from mice untreated or treated with PDT or with laser light alone were mixed with L1210 tumor cells in 0.1 ml PBS and then inoculated intradermally in mice.

PDT and adoptive immunotherapy. Spleen cells from PDT treated tumor bearing mice,or laser light treated tumor bearing mice or virgin mice were collected, washed and 20x106 cell/mouse were inoculated i.v. in recipient mice, pre-immunosuppressed with Cy (200mg/kg), as reported previously [18], and treated with PDT.

PDT + adoptive immunotherapy + chemotherapy. Spleen cells from PDT tumor treated tumor bearing mice, or laser light treated tumor bearing mice or virgin mice were collected, washed and 20x106 cell/mouse were inoculated i.v. in recipient mice at days +7. NVB or CDDP were inoculated i.p. at days +4. PDT was performed at days 5 and 6 after tumor injection. All the animals were immunosuppressed with Cy (200mg/kg) ad day –1 before the tumor transplantation.

Statistical analysis. The Mann-Whitney U-test was utilized to compare the survival times of the different groups [19].

Table 1. Immune lymphocytes cytotoxicity in vivo (winn assay)

IL	N° of mice with tumors	MST
$_{I}$N	5/5	11
IL	5/5	13
$_{I}$PDT	5/5	19*

Immune lymphocytes ($20x10^6$) mixed with L1210 cells (10^5) were inoculated i.d. on day 0
IN = normal spleen lymphocytes collected from virgin mice
IL = spleen lymphocytes collected from L1210 bearing mice pre-treated with laser light
$_{I}$PDT = spleen lymphocytes collected from L1210 bearing mice pre-treated with PDT
MST: Median Survival Time
*$p \leq 0.05$ by Mann-Whitney U test

Table 2. PDT + adoptive immunotherapy on l1210 leukemia

Treatment					
Day 0 L1210	day + 4 mTHPC mg/kg	day + 5 laser mW/ cm^2	day + 6 $_{I}$PDT	MST	D/T
10^6	-	-	-	11	5/5
10^6	0.3	100	-	16	5/5
10^6	-	100	$2x10^7$	15	5/5
10^6	-	-	$2x10^7$	16	5/5
10^6	0.3	100	$2x10^7$	24*	5/5

CDF1 mice, immunosoppressed with Cy (200mg/Kg i.p.) at day –1,challenged i.d. with 10^6 cells of L1210 leukemia
Foscan (mTHPC) : 0.3 mg/Kg
Laser light: 100 mW/cm^2 x 200 sec. of exposure (energy density: 20J/cm^2)
$_{I}$PDT: spleen lymphocytes collected from L1210 bearing mice pre treated with PDT, injected i.v.
*$p \leq 0.01$ by Mann-Whitney U test versus groups treated with only PDT, with only IPDT or with Laser light + IPDT

Results

The cytocidal efficacy of immune lymphocytes obtained from spleen of L1210 bearing mice pretreated with PDT, with Foscan and laser light, or with laser light only were tested in vivo Winn Assay. The spleen cells ($2x\ 10^7$) collected were mixed with 10^5 leukemic cells and than inoculated intradermally in syngeneic mice. We observed, in this experiment, that only PDT pretreated lymphocytes were cytotoxic in vivo against leukemic cells; in contrast the other lymphocytes population from laser light pretreated animals were ineffective like the controls animals injected with a mixture of normal lymphocytes and tumor cells at the same concentrations. In fact there is a statistically significance difference in median survival time among the different group of mice ,19 days versus 13 and 11 days (Table 1).

The photosensitizer utilize for PDT experiments is mTHPC, a second generation photosensitizers. The pharmacological activity of mTHPC is initiated by photoactivation with non-thermal light at a wavelength of 652 nm administered 24-96 hours following intravenous injection of mTHPC. Moreover it is removed more quickly by the body with less cutaneous photosensitivity. The therapeutic effect is mediated directly through the generation of highly reactive oxygen species, such a singlet oxygen, a process dependent on the intracellular interaction of mTHPC with light and oxygen. These free radicals are cytotoxic and disruptive to cells. Low doses of mTHPC (0.1-0.3 mg/kg) and light (10 J/cm2) are enough to obtain an optimal antitumor activity. The Foscan is considered 100-200 times more efficient than Photofrin in PDT. Foscan-PDT has received a European marketing authorisation for the palliative treatment of patients with advanced squamous cell carcinoma of the head and neck who have failed to prior therapies and are unsuitable for radiotherapy, surgery or systemic chemotherapy [20–23].

The antitumor activity of the combination PDT + adoptive immunotherapy with immune lymphocytes was evaluated in mice bearing L1210 leukemia (Table 2). Four days after tumor transplantation two groups of animals were treated with 0.3 mg/kg of Foscan and after 24 h the tumor masses were exposed to laser light (100mW/cm2 x 200" of exposure). These drug doses and light exposure were adopted in agreement with the optimal protocol obtained previously in our laboratory for other tumor models (18). One group was treated only with immune lymphocytes ($_{I}$PDT), collected from spleen of mice bearing L1210, pretreated with PDT(Foscan and laser light at the optimal doses). Another group of tumor bearing animals was treated at day five with laser light and 24hrs. later with immune lymphocytes. Finally one group of animals was treated with the combination PDT + adoptive immunotherapy.

The combined treatment modalities showed significant activity whereas adoptive immunotherapy alone and PDT alone had weak effect on this tumor The MST of the combination PDT + Immune lymphocytes was respectively 24 days compared to 15 and 16 days for mice treated with $_{I}$PDT alone or with light and PDT alone.

To enhance the antitumor efficacy we decided to adopt a politherapy anticancer protocol, using lower doses of cytotoxic drugs to restrict their toxic effects on normal host tissues, that could be very important in clinical oncology. The combined therapy Cisplatin (CDDP) or Navelbine (NVB) + PDT + Adoptive immunotherapy were utilized against the aggressive murine L1210 leukemia. In our treatment studies the two cytotoxic drugs used are representative of the main classes of compounds in common clinical use.

The Navelbine (NVB) compound is antimitotic drug able to block the mitosis of cancer cells, interacting with tubulin and disrupting microtubule function,particularly of microtubules that compose the mitotic spindle apparatus, leading to metaphase arrest.(24) We adopted the same protocol for PDT as described above and we treated at day 4 mice bearing L1210 with 1mg/kg of NVB (a very low dose not toxic). Other groups of animal were treated only with PDT,with NVB only

Table 3. Antitumor activity of the combination nvb, immune lymphocytes and pdt with foscan on l1210 leukemia

Groups	Treatment					MST	D/T
	Day 0 Tumor L1210	Day + 4 NVB mg/Kg	Day + 5 mTHPC mg/Kg	Day + 6 Laser light mW/cm^2	Day +7 $_I$PDT		
1	10^6	-	-	-	-	12 (9 – 14)	6/6
2	10^6	1	-	-	-	13 (11 – 17)	6/6
3	10^6	-	-	-	+	14 (10 – 16)	6/6
4	10^6	1	-	-	+	15 (11 – 18)	6/6
5	10^6	-	0.3	100	-	14 (12 – 18)	6/6
6	**10^6**	**1**	**0.3**	**100**	**+**	2/6	

CDF1 mice challenged i.d. with 106 cells of L1210 leukemia
Foscan (mTHPC) : 0.3 mg/Kg
Laser light : 100 mW/cm^2 x 200 sec. of exposure (energy density: 20J/cm^2)
NVB: Navelbine: 1 mg/kg
MST: Median Survival Time
D/T: Dead animals/Total
$_I$PDT: 2x107 spleen cells collected from PDT pretreated L1210 bearing animals

Table 4. Antitumor activity of the combination cddp, immune lymphocytes and pdt with foscan on l1210 leukemia

Groups	Treatment					MST	D/T
	Day 0 Tumor L1210	Day +4 CDDP mg/Kg	Day + 5 mTHPC mg/Kg	Day + 6 Laser light mW/cm^2	Day +7 $_I$PDT		
1	10^6	-	-	-	-	12 (9 – 14)	6/6
2	10^6	0.5	-	-	-	12 (12 – 16)	6/6
3	10^6	-	-	-	+	14 (13 – 18)	6/6
4	10^6	0.5	-	-	+	13 (11 – 15)	6/6
5	10^6	-	0.3	100	-	16 (14 – 18)	6/6
6	**10^6**	**0.5**	**0.3**	**100**	**+**	**1/6**	

CDF1 mice challenged i.d. with 106 cells of L1210 leukemia
Foscan (mTHPC) : 0.3 mg/Kg
Laser light : 100 mW/cm2 x 200 sec. of exposure (energy density : 20J/cm^2)
CDDP : Cisplatin
MST : Median Survival Time
D/T : Dead animals/Total
$_I$PDT: 2x107 spleen cells collected from PDT pretreated L1210 bearing animals

(1mg/kg), and with Immune lymphocytes ($_I$PDT) and NVB + immune lymphocytes ($_I$PDT). Finally we adopted the politherapy protocol with NVB at day 4 PDT at days 5 and 6 and $_I$PDT at day 7 after the tumor transplantation. The results observed in Table 3 show that the treatment regimen "PDT + Adoptive Immunotherapy + Chemotherapy" is highly effective against an aggressive metastatic tumor: in fact 2/6 animals survived indefinitely. The other treatment modalities are not statistically different from the controls.

Following these positive results we carried out experiments of combined therapy with another antiblastic drugs with different mechanism of action, using Cisplatin that is able to make activated species reacting with DNA,forming both intrastrand and interstrand cross-links (25) The protocol was the same as the experiment described above. Table 4 shows that the combination therapy "CDDP (0.5 mg/kg) + PDT + adoptive immunotherapy" is highly effective against the aggressive murine L1210 leukemia; only 1/6 mouse died with tumor. Also in this experiment the CDDP dose utilize is non toxic for the host tissue and the animals did not show any sign of toxicity.

The same positive results of combined treatments, with both antiblastic drugs, were obtained if the treatment schedule was opposite, before PDT and after NVB or CDDP. PDT, at the optimal therapeutic dose use against other murine tumors , was otherwise slightly active against the ascitic tumors L1210. However, when Drugs + PDT + adoptive immunotherapy were combined, the antitumor effects were strong. It is difficult to propose a satisfactory explanation for this enhancement. It may be connected to the sum of the damage induced by the different modalities on the cell membranes on the vasculature by free radical and molecular oxygen and immune effector cells , as we already observed [16]. PDT may serve as a debulking treatment leaving fewer tumor cells to be killed by cytotoxic drugs and immune lymphocytes.

There have been substantial advances in the understanding of the PDT-induced tumor specific immune reaction. This effect may not be relevant to the initial tumor excision, but may be important in attaining long-term tumor control. Tumor sensitised lymphocytes can, under reduce tumor burden, eliminate small foci of viable cancer cells that have escaped from PDT. PDT induction of antitumor immunity have similarities to the immune reaction induced by tumor inflammation caused by bacterial vaccines or some cytokines. The activity of tumor sensitised lymphocytes is not limited to the original PDT treated site but can include disseminated and metastatic lesions of the same cancer. Thus, although the PDT treatment is localized to the tumor site, its effect can have systemic attributes due to the induction of an immune reaction. PDT generated tumor sensitised lymphocytes can be recovered from distant lymphoid tissues (spleen, lymphonodes) at protracted times after light treatment. Therefore, it seems evident that these lymphocyte populations consist of immune memory cells [26]. The induction of immunity against a weekly immunogenic murine fibrosarcoma MS-2

by aluminum phthalocyanine-based PDT was also described [27]. Thus antitumor immunity fostered by PDT has a strong dependence on the activity of cytotoxic T cells.

These results demonstrate the generation of immune memory cells sensitized to PDT treated tumor and suggest that PDT may be particularly suitable for a combined application with adoptive immunotherapy protocols.

In conclusion, the interaction between PDT and cytotoxic drugs and immune lymphocytes may have important clinical implications and merits further investigation. In cancer treatment PDT could play a role in combinations of available therapies. It might be considered in a politherapy anticancer protocol using lower doses of cytotoxic drugs to restrict their toxic effects on normal host tissues and adoptive immunotherapy to induce an immune response.

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