RESPONSE OF MURINE MAMMARY ADENOCARCINOMA TO PHOTODYNAMIC THERAPY AND IMMUNOTHERAPY

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A murine mammary adenocarcinoma was treated with intraperitoneal photofrin II and laser (10 mW, 632 nm, He-Ne laser). Photofrin II was given intraperitoneally 4 h before 30-min laser treatment. A total of six photofrin II/laser treatments, given at 3–4 day intervals, resulted in 52.6% complete response, the cure rate was 26.3%; BCG or PD60 were given in association with PDT to CBA mice with mammary carcinoma. Addition of nonspecific immunostimulants significantly increased the antitumour effects of PDT, and the combination of PDT + PD60 resulted in the highest incidence of complete tumour regression (82.1%).

Enhanced activity of phytohemagglutinin-M response T-lymphocytes and natural killer cells capable of killing K562 tumour cells was noted.

These experimental observations i.e. marked inhibition of the mammary adenocarcinoma developed due the 'combined' PDT-immunotherapy, suggest the possible use of this new methodology as an adjuvant in clinical practice.

KEY WORDS Photodynamic therapy Immunotherapy Photofrin II He-Ne laser Murine mammary adenocarcinoma Immune response Phytohemagglutinin

Introduction

The increasing interest in photodynamic therapy (PDT) finds its origin in works of Dougherty *et al.*, performed on animal tumours.^{1,2} As a result, more than 3000 patients benefited from this new therapy method.^{2–4} Clinical utilization of photodynamic therapy is more advanced in U.S.A., Japan, Canada, Australia, China and Italy.³

In most clinical cases, hematoporphyrin derivative and photofrin II (PII) purified preparation are the most frequent photosensitizing agents in use for cancer photodynamic treatment, both in experimental studies^{5–12} and also in clinical trials.^{3,13} Mammary carcinoma represents a major problem and affects 1:14 women in U.S.A.¹⁴

It is a well known fact that vascular lesions appear immediately after photodynamic treatment. Most studies focused their attention on primary modifications which appeared in irradiated tissue structure and functions. We have few available information referring to modifications after two weeks of sole or immunostimulating agent associated photodynamic treatment. This fact is important for determining tissue tolerance as a consequence of PDT repeated treatments.

In the present work we have studied the effects of immunomodulating agents associated with PDT

upon the evolution of murine mammary adenocarcinoma.

Materials and Methods

Animals and Tumour

The tumour is a spontaneous arisen moderately differentiated CBA-IC₃₄ murine mammary adenocarcinoma. The tumour was propagated by serial transplantation. Large tumours were dissected under sterile conditions. Macroscopically viable tumour tissue was minced with scissors and forced through sterile needles of decreasing dimensions. Tumoural suspension (0.2 ml) was subcutaneously infected in the dorsal region of syngeneic animals. Tumours are visible in 7 days after transplant and have a volume between 75–108 mm³. In the beginning, the females were aged 8–10 weeks, weighing 19–24 g. The animals were from Cantacuzino-Institute farms and were kept in subdiurnal light, two per cage, under standard laboratory diet conditions and water ad libitum.

Photosensitizer

Photofrin II (Photofrin Medical Inc. Cheetkowage, NY) Mice were intraperitoneally injected at a drug dose of 15 mg/kg body mass. Four hours post-injection the mice were photoirradiated.

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Laser Phototherapy

Our light source was an expanded beam of a LG-6 He-Ne laser (632 nm; 10 mW) whose output was checked periodically with a Spectra Physics C powermeter.

Nonspecific Immunotherapy

BCG was obtained from Romanian BCG-Laboratory (Bucharest). The total dose utilized throughout the experiment was 1.5 mg BCG, suspended in 1.5 ml saline and peritumourally injected (0.5 mg/mouse) at 7 days interval. PD60 is a semipurified preparation extracted from *Streptococcus hemolyticus* (CI-12763-d) mutant (nonpathogenic and nonhemolytic) (Dima, 1990, figure 11). PD60 fraction was used as nonspecific immunostimulant in a total concentration of 1.2 mg/animal, distributed in six doses (200 µg/dose/animal) injected at 3–4 day intervals between doses.

Immunostimulating agents (BCG and PD60) were peritumourally administered, separately or in association with PDT, 24 h after photoirradiation.

Experimental Design

Six different modes of treatment were evaluated: PDT (33 J/cm²—in six fractionated exposures, 30 min each); BCG; PDT + BCG; PD60; PDT + PD60 and control mice (saline).

The mice were regularly assessed and tumour size was measured along three orthogonal diameters ($D_1 + D_2 + D_3$), every third–fourth day, in the first 2 weeks and then weekly till treatment end.

The volume was calculated assuming spheroid geometry:

$$\text{Vol} = (\pi/6)D_1 \times D_2 \times D_3$$

Immunological Tests

Mitogenic Response of Spleen Cells. Splenocytes from the six batches (by 15 animals—similarly to those from experimental design) and also a control batch consisting of 35 healthy animals (not inoculated) were fractionated on a nylon column according to the method of Julius *et al.*¹⁵ The resultant T-cell enriched cells (nylon wool-nonadherent) were cultured at a density of 2×10^5 cells/well in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine, 100 µg of streptomycin per ml, 100 units of penicillin G per ml, and 10% fetal calf serum (Gibco-Europe U.K. with or without addition of PHA (phytohemagglutinin-M, Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) in triplicate wells at 37°C in a CO₂ incubator (5% CO₂ + 95% air) for 48 h, 20 µl aliquots of [³H]-thymidine (20 Ci/mmol–12.5 µCi/ml; New England

Nuclear Corp. Boston, U.S.A.) in RPMI medium were added to the wells. After 48-h cultivation, the cells were harvested on glass filter papers (and washed with 5% trichloroacetic acid) methanol-dried, and then introduced in vials containing 10 ml scintillation liquid using 0.4% PPO (2,5-diphenyloxazole) and 0.01% POPOP (1,4 bis-(5-phenyloxazolyl)-benzene) solution in toluene. The radioactivity was read in a Beckman spectrometer.

Cytotoxicity Assay. Effector cells cytotoxicity was assayed by [³H]-uridine method as described previously by Nishimura *et al.*¹⁶ Briefly, aliquots (0.1 ml) of effector cell suspensions were mixed with 0.1 ml of [³H]-uridine (spect. act. 30 Ci/mmol—IFA, Bucharest, Romania) labelled target cells ($1.2 \times 10^5/0.1$ ml) in Coster 96-well round bottom plates at 200:1 effector to target ratios and cultured for 18 h at 37°C. The cells were harvested with the harvester and the retained radioactivity determined by a standard scintillation technique. Target cell lysis was calculated by the following equation: % cytotoxicity = (1-cpm in culture of effector and target cells/cpm culture of target cells alone) × 100.

Histology

Tumours were removed from animals (from animal groups used for the immunological tests) after two weeks of treatment (3 weeks post-transplantation) alone or combined; fixed in buffered formalin; sectioned and stained with hematoxylin–eosin.

Statistics

Data were expressed as arithmetic mean and standard error (S.E.). Significance of differences between groups was calculated by the Kruskal–Wallis test.

Results

Tumour Development and Mouse Mortality

Tumoural growth curves of the five batches receiving sole treatment (PDT, BCG, PD60) or associated therapy (PDT + BCG, PDT + PD60) are presented in Figures 1 and 2.

After 14 days of treatment (21 days post-transplant), tumoural volume values found in the five batches are under those of control batch (saline). In animals from batch I, exposed to photodynamic therapy (33 J/cm²), a slow growth of tumoural volume was noticed, compared to batch VI (saline). In mice from batch II, peritumourally injected only by BCG, was noticed a stagnation in tumoural growth, compared to tumoural volume values noticed in the other batches (excepting batch V).

Figure 1. The change in mean tumour volume in murine mammary adenocarcinoma injected with time after treatment. Both tumour-bearing and nontumour-bearing animals are included: — saline-treated control group; ---- He-Ne laser; BCG; - · - · - PDT + BCG

Figure 2. The change in mean tumour volume in murine mammary adenocarcinoma injected with time after treatment. Both tumour-bearing and nontumour-bearing animals are included: — saline-treated control group; ---- He-Ne laser; PD60; - · - · - PDT + PD60

After 45 days post-treatment, examination of tumoural volumes curves suggests two aspects: (1) singular treatment induced a partial delay of tumoural growth and tumoural volume values found in the three batches (PDT, BCG, PD60) were significantly lower in comparison with control batch (saline) (2) PDT association with BCG or PD60 led to an important decrease of tumoural volume and also to mortality and tumoural incidence diminishing (Figures 1 and 2, Table 1).

In animals exposed to photodynamic treatment, associated with streptococcal extract (PD60), the values of investigated experimental parameters (mortality, tumoural volume, progressive evolution and complete tumoural regression) were superior as compared to values noticed in the other batches. These facts demonstrate the efficacy of PDT treatment associated with PD60 in experimental mammary adenocarcinoma.

Mitogenic Response of Spleen Cells

Cpm mean values after lymphocytes stimulation with PHA, 14 and respectively 45 days after treatment, are presented in Table 2. After 14 days, we noticed a superior response post-PHA stimulation in all studied batches, as compared to values found in batch VI (saline) and healthy mice. Significant values were found in batches III and V, receiving associated treatment (PDT + BCG, PDT + PD60) (Table 2). Forty-five days post-treatment, cpm values found after lymphocytes stimulation with PHA were under those noticed after 14 days (Table 2), but above those in batch VI. Cpm values found in PHA-stimulated lymphocytes from animals in batch V (PDT + PD60) were significantly superior in comparison with the other batches (Table 2).

Table 1. Mortality data and tumour incidence

Treatment group	Percentage and number of dead mice during experiment*	Tumour incidence†	
		Percentage of mice with progressive tumour	Percentage of mice in which tumour regressed completely
I PDT	47.4 (9/19)*	15.8 (3/19)	26.3 (5/19)
II BCG	62.5 (15/24)	8.3 (2/24)	29.2 (7/24)
III PDT + BCG	20.0 (5/25)	28.0 (7/25)	52.0 (13/25)
IV PD60	33.3 (9/27)	29.6 (8/27)	37.0 (10/27)
V PDT + PD60	7.1 (2/28)	10.7 (3/28)	82.1 (23/28)
VI Control (saline)	91.3 (21/23)	8.7 (2/23)	—

* Numbers in parentheses denote the number of mice.

† Tumour incidence is based on the total number of mice alive in each group at 45 days after treatment.

Table 2. Results of lymphoproliferative assay in mammary adenocarcinoma of mice

Treatment group	14 days after treatment	45 days after treatment
	cpm after PHA stimulation	
I PDT	51 367 ± 1759	23 457 ± 608
II BCG	54 066 ± 428	22 897 ± 788
III PDT + BCG	62 542 ± 1576	34 440 ± 730
IV PD60	59 657 ± 1430	31 991 ± 1095
V PDT + PD60	76 118 ± 1104	45 307 ± 415
VI Control* (saline)	40 273 ± 2647	17 463 ± 901

* Healthy control animals: 38 927 ± 1035 cpm.

Cytotoxicity Assays

The results of cytotoxicity tests are presented in Table 3. Percentage values found for anti-K562 activity of spleen cells from the treated animals batch were superior to control values (saline), 14 days post-treatment, the following four groups: BCG, PDT + BCG; PD60 and PDT + PD60 had a significant anti-K562 activity, compared to the controls. The batch exposed to photodynamic treatment had superior cytotoxicity percentage values in comparison with control animals (saline) but inferior to healthy mice batch (Table 3). Forty-five days post-treatment, in all studied batches a decrease of splenic cells cytotoxicity was noticed, against K-562 target cells, in comparison with the values determined 14 days post-treatment. Anti K-562 cytotoxic activity, in irradiated batches and in those where immunotherapy was associated with BCG or PD60, was superior to the other animal batches (Table 3).

Histopathologic Examination

Morphological modifications appeared in tested animals and controls presenting mammary adenocarcinoma were studied two weeks post-treatment.

Table 3. Results of cytotoxicity assays using splenic effector cells from mammary adenocarcinoma injected mice

Treatment group	Percentage of cytotoxicity against target K-562 cells	
	14 days after treatment	21.8 ± 0.66 treatment
I PDT	20.3 ± 0.98	21.9 ± 0.66
II BCG	22.1 ± 0.96	20.2 ± 1.30
III PDT + BCG	25.4 ± 0.96	24.7 ± 0.35
IV PD60	24.9 ± 0.4	23.8 ± 0.79
V PDT + PD60	27.5 ± 1.08	29.5 ± 1.20
VI Control (saline)	17.2 ± 0.63	15.9 ± 0.62

Healthy control animals : 20.73 ± 0.95 cpm.

In animals from control batch (saline-injected) we noticed, in tumoural mass, necroses regions, hemorrhages (here and there), oedema, reactive inflammatory infiltrations, weakly differentiated carcinomatous cells, stroma with reduced reaction, interstitial hemorrhages and glandular structures, reflecting adenocarcinoma histological aspects (Figure 3).

In animals exposed to photodynamic treatment (33 J/cm²), tumoural proliferations are more restricted, under the form of regions with reduced dimensions, which present (inside), oedemas, hemorrhages and at tumour periphery vasodilatation appears in adipose tissue. In the profound layers of tegument, besides marked oedema, vasodilatation, hemorrhages, we also noticed leukocytic infiltrations (Figure 4).

In animals from batch II, treated with 1.5 mg BCG, peritumourally injected, histopathologic examination showed an extension of necroses regions in tumoural mass, oedema and leukocytic infiltration zones (Figure 5). Association of photodynamic therapy with BCG stressed out, at derm

Figure 3. Tumoural fragment in control mouse (not treated). Hematoxylin-eosin. $\times 1100$

level, carcinomatous lobuli presenting the tendency of disintegration and micronecroses, along with enriched stroma, vasodilatations, oedema and an inflammatory reaction (Figure 6).

In animals from batch V, exposed to photodynamic treatment associated with streptococcal extract (PD60) peritumoural injection, we noticed large hemorrhagic regions, marked vasodilatation, extended necroses zones in tumoural mass and especially inside these tumoural regions. Carcinomatous cells are still present only at the periphery of these regions. In Figure 7 we notice a carcinomatous lobulus, with central necrosis, substituted by leukocytic infiltrations, marked oedema, vasodilatation and tumoural parcelling. In Figure 8, we notice relatively restricted carcinomatous cells, as a consequence of necrosis, oedema, hemorrhage. Also, vasodilatation with a tendency of recent thrombosis, endothelial lesions and leukocytic infiltration.

Figure 4. Cutaneous fragment in mouse with He-Ne laser treated tumour. Hematoxylin-eosin. $\times 165$

Finally, histopathological lesions found 14 days after sole or BCG/PD60 associated photodynamic treatment were as follows: quantitative decrease of tumoural proliferations, necroses accentuation with leukocytic infiltrations inside, vasodilatation and variable oedema in hemorrhagic regions. Histopathological examination confirms the above-mentioned observations referring to immunologic response and reduced tumoural incidence in animals exposed to photodynamic radiation associated with nonspecific immunologic agents (BCG or PD60).

Discussion

In the present paper, as in previous ones,⁹⁻¹¹ we have noticed that photodynamic association with

Figure 5. Cutaneous fragment in mouse with BCG treated carcinomatous tumour. Hematoxylin-eosin. $\times 165$

Figure 6. Tumoural cutaneous fragment in photodynamic therapy + BCG-treated mouse. Hematoxylin-eosin. $\times 165$

immunotherapy significantly reduced mortality, volume and tumoural incidence in inbred CBA mice with mammary adenocarcinoma.

Treatment period covering 14 days (between 7 and 21 days post-transplant) was chosen that way because we noticed, in previous investigations, a significant response of immune system and an increased sensitivity to immunomodulation.

The value of studied experimental parameters, in the course of the 45 days from treatment initiation, emphasized variations of the immune response among animal batches and dependent on administered treatment. Tumoural incidence decrease, both as progression and as complete regression, is assigned to photodynamic therapy exposure but also to immunostimulators association, increasing cell-mediated immune response.

The results showed that 14 days after treatment initiation T-splenic lymphocytes significantly react to PHA stimulation, in comparison with healthy control animals. The cells giving the best response had their origin in mice receiving associated treatment (PDT + BCG and PDT + PD60). In animals exposed to PDT treatment associated with streptococcal extract—PD60, lymphocytes-T proliferation significantly increased after PHA stimulation (Table 2).

Splenocytes from animals exposed to bimodal treatment demonstrated an increased anti K-562 activity, compared to saline injected controls and healthy animals respectively, 14 days post-treatment. This increase of cytotoxic activity, noticed in our experiments, could be assigned to both stimulation with tumoural antigens and also to nonspecific

Figure 7. Tumoural cutaneous fragment in PDT + immunotherapy (PD60) treated mouse. Hematoxylin-eosin. $\times 550$

Figure 8. Tumoural cutaneous fragment in photodynamic therapy + immunotherapy (PD60)—treated mouse. Hematoxylin-eosin. $\times 550$

immunostimulating agents (BCG or PD60).

Moreover, we noticed that after PDT association with BCG or PD60, immunologic response was completely different: (a) after BCG peritumoural administration we noticed a partial improving both of investigated tumoural markers values and also of immunologic parameters. These observations can be explained by interferon induction and by temporary increase of NK cells activity. Some studies showed that prolonged BCG stimulation can induce suppression of T, NK cells and suppressor, NK cells and suppressor macrophages;^{17, 18} (b) photodynamic therapy association with streptococcal semipurified extract significantly improve tumoural and immunologic parameters under study (Figures 1 and 2; Tables 1–3). These effects could be assigned to the increase

of mitogenic activity, cytotoxicity, NK cells activity, interferon. Similarly effects were detected in other streptococcal preparations, too.^{19–22}

Histopathological modifications noticed in our experiments, after two weeks of singular or associated treatment administration, are in full agreement with previous observations of other authors referring to probable mechanisms which could act in photodynamic therapy.^{5, 6, 23–29}

Data presented in this paper demonstrate that exposure to PDT associated with immunotherapy stimulates antitumoural activity of photodynamic therapy and suggest that bimodal treatment reduced tumoural incidence (progressive evolution and complete tumoural regression) in murine mammary adenocarcinoma.

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