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Immunomodulatory effect of photodynamic therapy in patients with non-melanoma skin malignancies

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Photodynamic therapy (PDT) has been recognized as a noninvasive and new therapeutic approach to the effective treatment of tumors. It has been shown in studies concerning malignant cell lines and various animal tumor models that the interaction of photosensitizing substances with light leads to the release of cytotoxic substances and stimulates the immune response. The aim of our study was to analyze the immune system activation in patients with basal cell carcinoma (BCC) during the photodynamic therapy. Patients with skin malignancies were treated topically with 10% δ-aminolevulinic acid (ALA) (Medac GmbH, Wedel, Germany). The light came from an argon -pumped dye laser. Blood samples were collected from each patient twice a day: before and after the photodynamic treatment procedure. The study was conducted on lymphocytes and granulocytes from peripheral blood. In the cell culture supernatants, the concentration of tumor necrosis factor α (TNFα), the percentile composition of patients' lymphocytes and the chemiluminescence of neutrophils were measured. We have observed an increase in the intensity of the chemiluminescence, an increase in TNFα concentration and the insignificant change in lymphocyte percentile composition.

1. Introduction

Photodynamic therapy (PDT), which means a systemic or local (topical) administration of photosensitizers selectively absorbed in malignant tissue, followed by irradiation with laser light, is a new, selective, noninvasive and highly efficient therapeutic method [1]–[3].

Destruction of a tumor is connected not only with the direct cytotoxic effect of laser energy, but is also triggered by PDT reaction of photodynamic oxygenation,

including free radicals generation and, in effect, selective destruction of malignant tissue [1], [2], [4].

Stimulation of the immune response after treatment with laser light and photosensitizers has been shown in a number of experimental situations, including *in vitro* studies concerning malignant cell lines and various animal tumor models [5]. Results of these experiments confirm the role of immune cells and their mediators in the destruction of malignancies [6]–[10]. This process is usually connected with local inflammation.

The purpose of this study was to analyze the immune system activation during the photodynamic therapy in patients with basal cell carcinoma (BCC).

2. Patients and methods

Patient population. 10 patients participated in our study (8 women and 2 men, average age 68 years, range: 47–81 years). Patients were informed of the research character of their examination and signed a consent form. The Ethical Committee of the Silesian Medical University approval was obtained for their treatment. PDT was either primary or recurrent after previous therapy (cryosurgery, surgical excision). A total of 10 patients with 12 sites were treated, 6 with ulcerating BCC, 3 with nodular BCC and 1 with superficial BCC.

Nine of the 12 treated lesions were located on the face, 2 lesions on the trunk and 1 lesion on the leg.

Biopsies were taken from all lesions prior to PDT. In the patients with multiple lesions, a biopsy was performed on a single lesion.

Drug application. In our study δ-aminolevulinic acid (ALA) (Medac GmbH, Wedel, Germany) as a precursor in the biosynthesis of haem was used. A thin layer of an oil-in-water emulsion containing 10% ALA and 20% dimethyl sulphoxide (DMSO) was applied topically to the tumor with a 4-mm margin of surrounding normal tissue.

PDT light source. An argon pumped dye laser (Coherent Inc., Palo Alto, CA, U.S.A.) tuned to emit radiation at 635 nm was used for the light treatment. Irradiation was done with the vendor-provided fiber optic ended with microlens defocusing collimated laser light by 30°, giving thereby a homogenous circular field covering the treated lesion. Laser radiation emitted from the fiber was monitored with a power meter (Coherent Inc., Palo Alto, CA, U.S.A.). The energy density of the laser irradiation was 100 J/cm^2.

Treatment procedure. An oil emulsion containing 10% ALA was applied topically to the lesion sites. The sites were then occluded with a self-adhesive foil for 9 hours. Before the light treatment, the tumor fluorescence was assessed visually with LIFE system (Xillix Technologies Corp., Richmond, BC, Canada) which uses for excitation helium-cadmium laser light (442 nm). Tissue accumulation of protoporphyrin IX (PpIX) was viewed as a red fluorescence. Irradiation was performed in several [6]–[8] sessions using the light from an argon-pumped dye laser with a wavelength of 635 nm

giving a radiation dose of 100 J/cm^2 per session. We included at least a 10–20% margin around lesions in the field of irradiation. The treatment time was about 10 min, at a low fluence rate and interrupted periodically during the treatment, with a 2-hour interval. All patients were reviewed at two-week intervals and treatments were repeated if required.

Follow-up study. The post-treatment observation period ranged from 10 to 19 months. One recurrence of nodular BCC during 6 months was observed.

Evaluation. A complete response was achieved in 1 patient with superficial BCC, in 6 patients with exulcerans BCC and in 2 patients with nodular BCC. A partial response after PDT was observed in 1 patient with nodular BCC. One recurrence during 6 months was observed in nodular BCC (Tab. 1).

Table 1. Tumor response.

Tumor type	Patients	Complete response	Partial response	Recurrence
Superficial BCC	1	1		
Nodular BCC	3	2	1	1
Exulcerans BCC	6	6		

Adverse reaction. The treatment was well tolerated by most patients. Moderate pain during the treatment of localized erythema and edema was observed immediately after the light treatment and after 1–2 days.

Blood samples. Blood samples were collected from each patient twice a day: before and after the photodynamic treatment procedure. The study was conducted on lymphocytes and granulocytes from peripheral blood, isolated by means of a double density gradient made up of Histopaque solutions of 1.007 and 1.119 g/cm^3 (Sigma Chemical Company, St. Louis, MO, U.S.A.). Neutrophils were washed and resuspended in Hank's buffered saline solution; lymphocytes were washed and resuspended in a culture medium.

Lymphocyte culture. Lymphocytes were incubated for 72 h at 37 °C and 5% CO_2/air in a culture medium consisting of: 10 ml Heat Inactivated Foetal Bovine Serum + 2.5 ml Hepes Buffer + 1 ml antibiotic solution (Penicillin + Streptomycin) + 100 µl 2-Mercaptoethanol filled to 100 ml with RPMI 1640 Medium. Cells were treated with phytohaemaglutinin (PHA), concanavalin-A (Con-A) and lypopolisacharide (LPS). Untreated lymphocytes served as a control. We used sterile four-wells Nunc plates. The final concentration in wells was: 10^6 cells, 10 µg/ml PHA, 5 µg/ml Con-A, 100 ng/ml LPS. In the control well the mitogen was substituted by an equal volume of the culture medium. The final volume in each well was 1 ml.

After 72 h of incubation, cells were removed by centrifugation and supernatants were stored at –20 °C.

TNFα concentration. In the cell culture supernatants the concentration of TNFα was established using Human TNFα Immunoassay Quantikine Colorimetric Sandwich ELISA (R&D, Minneapolis, MN, U.S.A.) and a microplate reader Elx 800 (BioTek

T a b l e 2. TNFα concentration in cell culture supernatants after PHA-stimulation and without mitogen stimulation (control).

	TNFα concentration [pg/ml]			
	After PHA-stimulation		Without stimulation (control)	
Patient number	Before PDT	After PDT	Before PDT	After PDT
4	925	1153	24	155
11	1116	1686	109	0
12	219	3976	0	0
14	6100	6700	0	1
15	2545	3630	55	50
18	4310	4430	129	175
19	1572	5350	142	162
22	5300	6350	592	658
23	2803	4735	483	732
28	2125	2425	25	130
Average	2701.5 ± 1954	4043.5 ± 1868	155.9 ± 209	206.3 ± 267
	$p = 0.134$ (NS*)		$p = 0.645$ (NS)	

*NS – not significant.

Instruments Inc., Winooski, VT, U.S.A.). We decided to use the supernatants after PHA-stimulation and the untreated cell cultures (control) to measure TNFα levels. The obtained results are presented in Tab. 2.

Lymphocyte percentile composition. We determined the percentile composition of patients' lymphocytes. The lymphocyte sub-populations of $CD3^+$ (T-lymphocytes),

T a b l e 3. Lymphocyte percentile composition.

	$CD3^+$ lymphocytes [%]		$CD4^+$ lymphocytes [%]		$CD8^+$ lymphocytes [%]		$CD16^+$ lymphocytes [%]		$CD19^+$ lymphocytes [%]	
Patient number	Before PDT	After PDT	Before PDT	After PDT	Before PDT	After PDT	Before PDT	After PDT	Before PDT	After PDT
4	56	47	49	39	11	9	12	4	13	3
11	53	72	46	42	6	6	5	9	1	1
12	62	44	45	35	11	11	19	5	8	2
14	44	65	33	38	21	23	10	13	3	6
15	66	57	36	39	20	23	22	19	9	8
18	70	61	46	53	15	22	16	20	14	8
19	56	54	42	39	11	20	15	16	8	16
22	56	69	33	40	26	21	27	19	9	11
23	65	70	37	49	17	34	10	20	14	15
28	52	50	43	50	14	15	21	20	10	3
Average	58 ± 8	59 ± 10	41 ± 6	42 ± 6	15 ± 6	16 ± 8	16 ± 7	15 ± 6	9 ± 4	7 ± 5
	$p = 0.825$ (NS)		$p = 0.604$ (NS)		$p = 0.335$ (NS)		$p = 0.686$ (NS)		$p = 0.471$ (NS)	

$CD4^+$ (inductive and helper lymphocytes), $CD8^+$ (supressory and cytotoxic lymphocytes), $CD16^+$ (natural killers) and $CD19^+$ (B-lymphocytes) were labeled with monoclonal antibodies, using the immunoenzymatic technique of Alkaline Phosphatase-Anti-Alkaline Phosphatase LSAB-2 Visualization Kit (DAKO, Glostrup, Denmark). The obtained results are shown in Tab. 3.

Neutrophil chemiluminescence. Chemiluminescence of neutrophils was measured after their stimulation with Phorbol Myristate Acetate (PMA, Sigma, St. Louis, MO, U.S.A.), added 5 min after the application of luminol, and recorded in a system equipped with a photomultiplier 9514s (EMI, Middlesex, U.K.). The sample volume was 1 ml. The following reagents were added to the neutrophils (10^6 cell/ml): luminol 5.6×10^{-6} M, PMA 1.6×10^{-7} M and Hanks buffer pH = 7.4 (GIBCO, Paisley, Scotland). Measurements of the chemiluminescence were performed by the technique of single photon counting, continuously for 15 min. The obtained results are presented in Tab. 4.

T a b l e 4. Neutrophil chemiluminescence [photons/15 min].

Patient number	Before PDT	After PDT
4	281470	717540
11	2105020	3830740
12	1031040	1794240
14	3518440	4519440
15	1010790	1158620
18	342840	1758220
19	1578920	1248460
22	1450570	1350450
23	807430	1037680
28	1134420	1651155
Average	1.326.094 ± 945.326	1.906.655 ± 1.251.663
	$p = 0.258$ (NS)	

For each patient, the peripheral blood morphology including percentile composition of leukocytes was determined. The following results were obtained: erythrocytes (RBC) – 4.46 ± 0.34 M/μl, hemoglobin – 13.65 ± 1.32 g/dl, hematocrite – 40.75 ± 4.04%, leukocytes (WBC) – 7.14 ± 1.68 K/μl. Percentile composition of leukocytes: neutrophils – 53.0 ± 7.8%, lymphocytes – 33.7 ± 6.1%, monocytes – 8.5 ± 1.2%, eosinophils – 3.7 ± 2.3%, basophils – 1.0 ± 0.5%. The results were stable during the photodynamic therapy. Comparison between results was performed according to the *t*-Student test.

3. Results and discussion

In the present study we observed an increase in the intensity of the chemiluminescence, suggesting polymorphonuclear cell activation during the photodynamic therapy.

Similar observations were reported by other authors as the result of *in vitro* studies [11]. Also with animal experimental models, the massive invasion of myeloid cells and their adherence in blood vessels after PDT was observed [10], [12]. Stimulation by a granulocyte-macrophage colony-stimulating factor amplifies a PDT-induced antitumor reaction [13]. The mobilization of human neutrophils and their cytotoxic potential is an important anticancer mechanism which participates in the tumor destruction process. Many environmental factors stimulate granulocytes to respiratory burst and degranulation. Activation leads to an increased cell oxygen uptake, to the production of reactive oxygen and reactive oxynitrogen intermediates. Extracellular release of oxygen radicals and proteolytic enzymes leads to tissue destruction [14]–[17].

The selected chemiluminescence measurement, based on registration of light emitted by activated cells, is the most appropriate method to detect living granulocytes activity and a good indicator of their free radicals production [18].

The increase in the intensity of the chemiluminescence may be the result of neutrophils' pre-activation, called "priming". One of the essential priming stimulating agents is tumor necrosis factor α, inducing human neutrophils' cytotoxic activity against malignant cells [19], [20]. The enhancement of fagocytosis and respiratory burst and the increase of integrin receptors' expression occurs in human polymorphonuclear cells after TNFα stimulation [21]. We suggest that the increase in the chemiluminescence observed in patients after the photodynamic treatment procedure, may be the result of neutrophils' priming, leading to their activation and ability to intensify multidirectional reaction for another stimulus [22].

In all analyzed lymphocytes culture supernatants of the patients, we recorded an increase in TNFα concentration. Tumor necrosis factor α is a cytokine produced by many types of cells, such as macrophages, monocytes, neutrophils and activated lymphocytes. The anticancer activity of TNFα is connected not only with the direct cytotoxic effect on target cells, but also with the damage of tumor vasculature, proinflammatory potential and mobilization of many immune cells via their surface receptors [23].

The mechanism of the TNFα direct cytotoxic effect is not clear and depends on the type of target cells. The tumor necrosis factor can induce necrotic or apoptotic cell death through different biochemical pathways which can lead to intranucleosomal DNA fragmentation [24]. In animal tumors after the injection of TNFα, haemorrhagic necrosis was observed, due to a cytokine attack on the tumor microvasculature, resulting in regression [23]. The TNFα modulatory effect is connected not only with direct influence on immune cell proliferation, but also with its indirect activation through other cytokine production and changes in the extracellular matrix [23], [25]–[28].

We find the increase in TNFα secretion by immune cells, observed during the photodynamic therapy, to be one of the positive immunomodulatory effects of PDT. The decrease in TNFα serum concentration after the photodynamic treatment, reported

by other authors, suggests the massive tumor infiltration by activated cells releasing TNFα, or an augmentation of the number of TNFα receptors [29].

The insignificant change in lymphocyte percentile composition observed in the present study is probably the result of the local immune response during PDT. The dermal inflammation associated with the photodynamic therapy of basal skin carcinoma is usually limited and involves only the tumor region. It is an important benefit of PDT, which makes the photodynamic treatment an alternative for conventional therapy of skin malignancies. Our results are parallel to those of other authors, confirming massive infiltration of the immune cells of a tumor and their local activation [10], [12]. During the photodynamic therapy of BCC these activated cells change the extracellular tumor matrix by cytokines and induce malignant cell destruction. In the present study we confirm neither the increased number of NK-cells after PDT reported by some authors working on animal tumor models [6], nor the cytotoxic lymphocyte percentile changes observed in patients suffering from *mycosis fungoides* [30].

In the present study, a high degree of deviations of analyzed parameters was noted what inevitably entailed no significant statistical differences ($p > 0.05$). It is probably the result of the small number of patients in the analyzed group and also of the considerable differences in their immune system reactivity associated with their age (47–81 years) and many concomitant diseases demanding therapy interfering with the immune response.

Further studies on the immunomodulatory effect of the photodynamic therapy in patients with basal cell skin carcinoma are in progress.

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