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Antiproliferative, Antiangiogenic and Apoptotic Effect of Photochemotherapy (PUVA) in Psoriasis Patients

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A B S T R A C T

The aim of the study was to investigate the antiproliferative, antiangiogenic and apoptotic effect of photochemotherapy (PUVA) in psoriatic patients, and to compare it with a control group of psoriatics treated with local corticosteroid therapy. The study included 60 psoriasis patients, 30 of them allocated to PUVA therapy and local corticosteroid each. Immunohistochemical methods of staining with Ki-67, F-8 and bcl-2 antibodies were used to determine proliferative keratinocyte count, to visualize the number of blood vessels in the dermis, and to determine the number of cells exhibiting expression of the antiapoptotic oncoprotein bcl-2, respectively. In all study patients, the values of Ki-67, F-8, bcl-2 and PUVA score were recorded pre- and at six weeks post-therapeutically. Study results showed a statistically significant decrease in the epidermal proliferative keratinocyte count and dermal number of blood vessels after both therapeutic modalities ($p<0.001$ both). The value of bcl-2 showed a statistically significant increase in the group of patients treated with PUVA therapy ($p=0.001$) and an increase in the control group, demonstrating enhanced keratinocyte apoptosis after treatment. Accordingly, study results demonstrated the antiproliferative, antiangiogenic and apoptotic effect of both PUVA and local corticosteroids. These very mechanisms appear to play a key role in the action of most antipsoriatic therapies.

Key words: *Ki-67, F-8, bcl-2, PUVA, psoriasis*

Introduction

According to current concepts, psoriasis is an autoimmune, T-lymphocyte induced, cytokine and chemokine mediated disease[1]. Psoriasis is characterized by hyperproliferation and resistance of keratinocytes to apoptosis[2]. Vascular lesions in psoriasis include vascular dilatation and tortuosity in papillary dermis, and angiogenesis[3]. Angiogenesis is a key component of the psoriatic process promulgated by a variety of cytokines and angiogenic growth factors. These lesions are highly relevant, since increased intralesional microcirculation may precipitate the passage of T-lymphocytes to the skin, thus endothelial venules playing an important role in the genesis of psoriasis[4]. Apoptosis is the general name for physiologic cell death caused by activation of an energy-dependent suicide program[5,6]. In psoriasis, an increased apoptosis has been observed in the differentiated layers. It has been considered as a counteracting factor of cell overproduction by the germinative compartment[7,8]. The exact cause of psoriasis remains unknown, therefore the treatment is symptomatic. PUVA (psoralen + ultraviolet A) has for years been used in the treatment of psoriasis vulgaris involving more than 30% of the skin area, but its mechanism of action has not yet been fully elucidated. The aim of the study was to investigate the antiproliferative, antiangiogenic and apoptotic effect of PUVA in psoriasis patients by immunohistochemical analysis using Ki-67, F-8 and bcl-2 antibodies, and to compare it with the action of topical corticosteroids as a control group[9–12].

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Materials and Methods

The study was carried out at University Department of Dermatology and Venereology, Zagreb University Hospital Center in Zagreb. Sixty patients diagnosed with stable chronic plaque psoriasis gave their informed consent in writing and were randomized into two treatment groups. Experimental group included 30 patients with chronic plaque psoriasis involving more than 30% of the skin area, with indications for PUVA therapy. Control group included 30 psoriasis patients with indications for local corticosteroid therapy. In all patients, psoriasis skin involvement was evaluated according to the Psoriasis Area and Severity Index (PASI) score[13] before and after treatment. All patients had discontinued topical antipsoriatic medication for at least 4 weeks and any systemic antipsoriatic medication for at least 8 weeks prior to pretrial assessment. Before patient inclusion in PUVA group, complete blood count, renal and hepatic function tests, and ophthalmologic examination were performed in each patient, and those having normal results were enrolled in the study. PUVA therapy was administered according to the European protocol four times weekly for five weeks with 20 exposures *per* patient in total[14]. According to the protocol, experimental group patients were administered the oral photosensitizer 8-methoxypsoralen in a dosage of 0.6 mg/kg, followed by ultraviolet A ray (UVA) irradiation after two hours. Control group patients applied a thin layer of corticosteroid ointment (betamethasone-diproprionate) exclusively onto psoriatic lesions twice daily. Biopsy specimens were obtained from lesional skin before treatment and from the same lesion after the treatment in both patient groups. All biopsy specimens were analyzed immunohistochemically (cell immunophenotyping). Paraffin block 3-μm sections were used on immunohistochemistry staining. Sections were stained by the standard immunoperoxidase avidin--biotin method on an automated staining device (TehMate, Dako, Glostrup, Denmark) using primary antibodies (anti-Ki-67, anti-F-8, and anti-bcl-2) from the same manufacturer. The specimens were deparaffinized, buffer treated in a microwave oven (EDTA, pH 9.0, 15 min) to enhance antigen reaction, and stained in an automated device using capillary activity. Antibodies were used in 1:100 dilution. The reaction was visualized by use of diaminobenzidine (DAB, Dako, Glostrup, Denmark). Stained preparations were analyzed under light microscope. Proliferative index of the epidermis cells was expressed as percentage of positive anti-Ki-67 antibody stained cells. Positive reaction is nuclear cell staining. Anti-F-8 antibody staining revealed vascular endothelial cells in the skin specimens. Thus visualized blood vessels were counted *per* mm^2 specimen using the microscope mesh determining the given area. In this way, the absolute number of dermis blood vessels was shown in the respective skin specimens. Positive reaction on epidermal keratinocytes as well as membrane and cytoplasmic staining were visualized by anti-bcl-2 antibody staining. Positive cells were counted under a light microscope and expressed as number of positive cells *per* 100 counted cells (%). The Ki-67, F-8, bcl-2 and PASI score evaluation was performed at baseline and was repeated at 6 weeks of either therapy.

Statistical data analysis was done by description of all groups and all characteristics. The significance of therapeutic effect, i.e. differences between pretherapeutic and post-therapeutic condition, was determined by t-test for dependent samples (paired samples t-test).

Results

According to sex distribution, there were 53.3% of male and 46.7% of female patients in both experimental and control group. The parameters of proliferative, angiogenic and antiapoptotic activity showed a statistically significant post-therapeutic decrease in both patient groups treated with PUVA therapy and local corticosteroid therapy (Table 1). Comparison of changes in the parameters of antiproliferative, antiangiogenic and apoptotic effect between the groups of patients treated with PUVA therapy (Fig. 1) and corticosteroids (Fig. 2) showed the two treatments to have similar effects on Ki-67. The mean pretherapeutic and post-therapeutic value of Ki-67 was 50.0 and 32.3 in the experimental group *versus* 58.4 and 34.1 in the control group (Tables 1 and 2). A similar pat-

TABLE 1
PARAMETERS OF ANTIPROLIFERATIVE, ANTIANGIOGENIC AND APOPTOTIC EFFECT OF PUVA THERAPY AND RESULTS OF T-TEST FOR DEPENDENT SAMPLES

Variables		N[a]	X	*s*	t	df	p
Ki-67	Before therapy	30	50.0	25.08	4.74	29	<0.001
	After therapy	30	32.3	21.81			
F-8	Before therapy	30	25.0	12.00	5.70	29	<0.001
	After therapy	30	14.7	9.24			
bcl-2	Before therapy	30	16.0	13.40	–3.83	29	0.001
	After therapy	30	24.0	16.88			
PASI score	Before therapy	30	44.6	8.72	15.46	29	<0.001
	After therapy	30	13.4	6.99			

[a]Number of cases

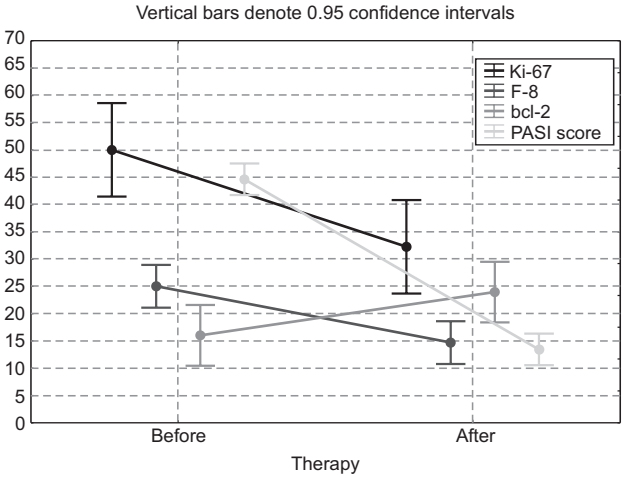

Fig. 1. Changes in the parameters of antiproliferative, antiangiogenic and apoptotic action following PUVA therapy (n=30).

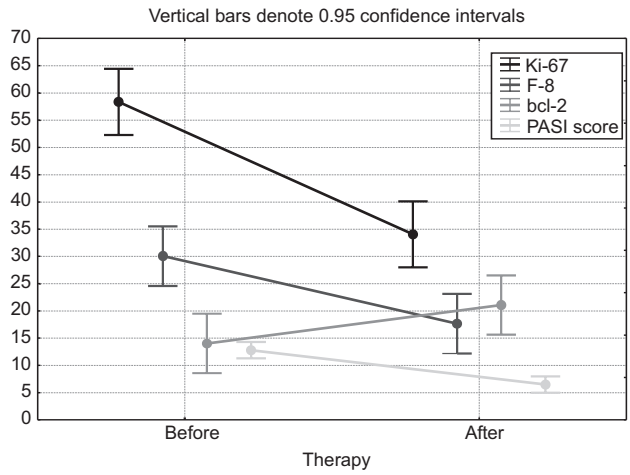

Fig. 2. Changes in the parameters of antiproliferative, antiangiogenic and apoptotic action following local corticosteroid therapy (n=30).

tern was also observed in the changes recorded in F-8 and bcl-2 (Tables 1 and 2, Figs. 1 and 2). Differences between the pretherapeutic and post-therapeutic mean PASI score in the two patient groups are depicted in Figs. 1 and 2. In the PUVA therapy group, the mean pretherapeutic and post-therapeutic PASI score was 44.6 (SD=8.72) and 13.4 (SD=6.99), respectively (Table 1). In the control group, the respective PASI score values were 12.8 (SD= 2.92) and 6.5 (SD=5.01) (Table 2, Figs. 1 and 2).

Discussion

Topical treatment of psoriasis includes corticosteroids, vitamin D analogs, dithranol (anthralin) and retinoids. Systemic therapy includes photochemotherapy (PUVA), retinoids, methotrexate, immunosuppressants and biologicals. PUVA is the most widely used systemic antipsoriatic therapy and its efficacy has been largely documented. PUVA has an antiproliferative and immunosuppressive action[15]. In PUVA treated psoriatic skin, the numbers of epidermal and dermal CD3+T-lymphocytes as well as of CD4, CD8 and IL-2 receptor+subsets are strongly reduced[16]. Peripheral blood mononuclear cells obtained from psoriatic patients before and upon completion of PUVA therapy showed inhibition of the production and release of interleukin-1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumor necrosis factor-α (TNF-α)[17]. The number of Langerhans cells is reduced after PUVA, although remission of psoriasis seems to be independent of the magnitude of this effect[18,19]. PUVA has no measurable effect on complement components and immunoglobulin levels in psoriatic patients[20]. In spite of all these facts and its longstanding use, the mechanism of action of PUVA therapy on psoriatic skin has not yet been fully elucidated[21]. Considering the above mentioned new concepts on the pathogenesis of psoriasis, we investigated the antiproliferative, antiangiogenic and apoptotic action of PUVA, and compared it to local corticosteroid therapy and clinical picture of the disease. PUVA can reverse the pathologically altered pattern of keratinocyte differentiation markers and reduce the number of proliferating epidermal cells[21]. The Ki-67 protein is essential for cell

TABLE 2
PARAMETERS OF ANTIPROLIFERATIVE, ANTIANGIOGENIC AND APOPTOTIC EFFECT OF CORTICOSTEROID THERAPY AND RESULTS OF T-TEST FOR DEPENDENT SAMPLES

Variables		N[a]	X	*s*	t	df	p
Ki-67	Before therapy	30	58.4	15.41	6.12	29	<0.001
	After therapy	30	34.1	17.65			
F-8	Before therapy	30	30.1	16.59	5.25	29	<0.001
	After therapy	30	17.7	13.20			
bcl-2	Before therapy	30	14.0	12.40	–2.14	29	0.041
	After therapy	30	21.1	17.09			
PASI zbroj	Before therapy	30	12.8	2.92	6.58	29	<0.001
	After therapy	30	6.5	5.01			

[a]Number of cases

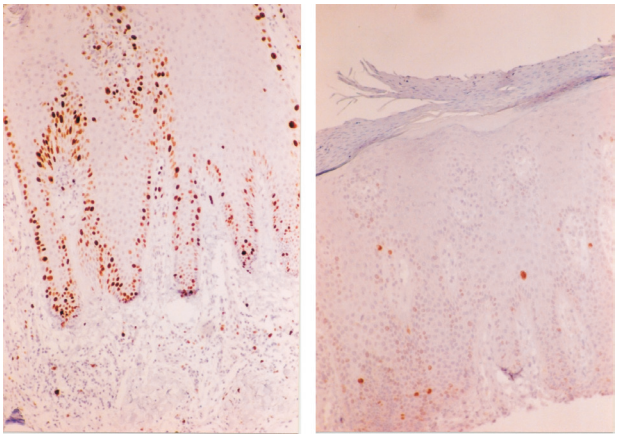

Fig. 3. A psoriatic skin specimen before and after PUVA therapy; anti-Ki-67 antibody staining (background staining with hemalaun, x200).

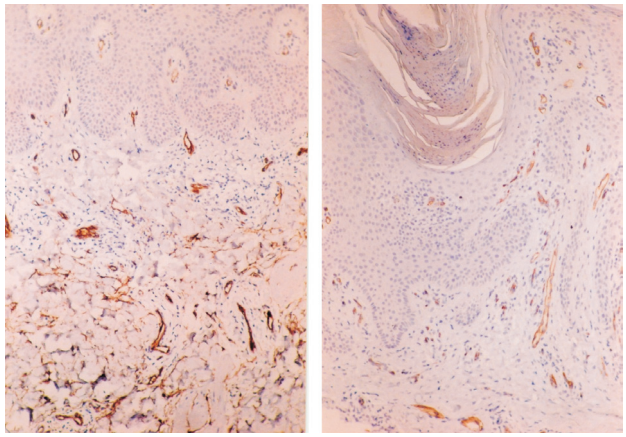

Fig. 4. Psoriatic skin before and after PUVA therapy; anti-F-8 antibody staining (background staining with hemalaun, x200).

proliferation and has been widely used as a proliferation marker[9,10]. In psoriasis, the expression of proliferative proteins is increased[9,21,22]. In the present study, the proliferating keratinocyte count was determined by use of Ki-67 antibody before and after PUVA therapy (Fig. 3) and local corticosteroid therapy. The levels of Ki-67 expression decreased after both treatments. These findings confirmed the expected antiproliferative effect of PUVA and steroid therapy in psoriatic lesions, which is consistent with literature data[23,24]. To our knowledge, there are no literature reports on the assessment of the proliferative effect of PUVA therapy by this immunohistochemistry method using the proliferation factor Ki-67. Hannuksela-Svahn *et al.* investigated p53 expression before and after PUVA therapy, with immunohistochemical determination of proliferation by use of Ki-67 in psoriatic and non-lesional skin before and after PUVA therapy; their results were consistent with those reported herewith[21].

Although psoriasis is primarily a lymphocyte driven disease, the prominence of dermal microvascular expansion in lesional skin suggests that psoriasis is angiogenesis dependent[25]. As early as 1972, Folkman identified vasoproliferation as a suitable target for the development of anti-psoriatic drugs[26]. His concepts proved correct, since currently there is evidence for the antiangiogenic effect of various antipsoriatic treatments in use, while research into new antipsoriatics is just under way[27–29].

In the present study, we investigated the number of blood vessels in the psoriatic lesions dermis before and after PUVA (Fig. 4) and local corticosteroid therapy. Endothelial cells were stained using monoclonal anti-F-8 antibody. The value of F-8 showed a statistically significant decrease ($p<0.01$) in both patient groups, i.e. both therapies led to a decrease in the number of blood vessels within the treated plaques.

Results of the present study demonstrated the antiangiogenic action of both PUVA and local corticosteroid therapy, confirming the concept of angiogenic activity as one of the key mechanisms in the management of psoriasis[3,10].

Braverman and Sibley studied the relation of vascular changes to epidermal changes in psoriatic skin undergoing PUVA. The degree of epidermal proliferation was quantified by autoradiography, while the state of dermal capillaries was monitored ultrastructurally by electron microscopy. As a result, they suggest that the response to PUVA therapy is mediated *via* microvasculature rather than any antiproliferative effect on basal keratinocytes[30]. They also conducted a study in psoriatic patients treated by Goeckerman's method, with similar results[30]. In Croatia, the antiangiogenic effect of naphthalene was demonstrated by Vržogić *et al.*[31], by use of immunohistochemistry methods of vascular staining, also employed in the present study.

Determination of the number of blood vessels by immunohistochemical labeling of endothelium using F-8 antibody as well as determination of vascular endothelial growth factor (VEGF) in blood[32,33] can be used as a reliable indicator of the clinical course of the disease and therapeutic effect by acting upon neoangiogenesis.

Keratinocyte apoptosis in psoriasis remains an inadequately investigated and poorly elucidated process in which many genes and their products are involved. Among them, the bcl-2 gene plays a major role. The expression of bcl-2 is restricted to cell populations with a long lifespan or proliferative activity, and it prevents apoptosis[6]. In normal skin, the expression of bcl-2 gene is present in the basal layers of the epidermis[34–36]. In psoriatic skin, a decreased expression of bcl-2 relative to normal skin has been demonstrated[37–39].

In the present study, the apoptotic effect of PUVA and corticosteroid therapy on keratinocytes was investigated by use of bcl-2 antibodies. A statistically significant increase of bcl-2 value was recorded in both PUVA treated group (Fig. 5) and control group of patients. These results suggested that in addition to lymphocytes[40,41], PUVA therapy also induced apoptosis in keratinocytes, and that the psoriasis regression following PUVA therapy was also related to the enhanced keratinocyte apoptosis in the basal layer of the epidermis. In our study, bcl-2 ex-

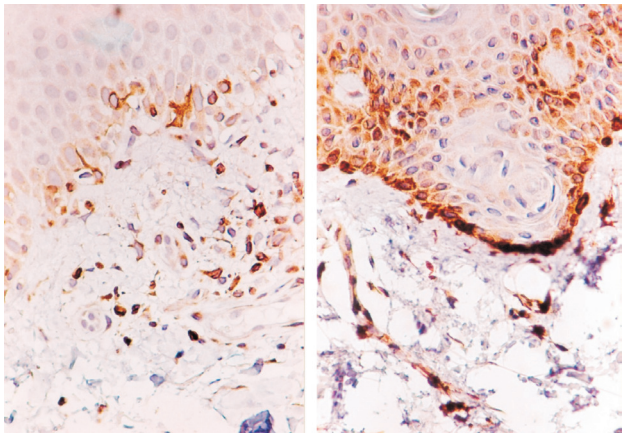

Fig. 5. Psoriatic skin before and after PUVA therapy; anti-bcl-2 antibody staining (background staining with hemalaun, x400).

pression was observed in keratinocytes obtained from all over the epidermis, without isolation of particular layers. Basal layer keratinocytes showed highest bcl-2 expression, whereas most terminally differentiated keratinocytes from the upper layers of the epidermis showed no cytochemical staining at all, i.e. no bcl-2 expression. Only melanocytes and cells of the basal layer of the epidermis, i.e. dividing cells, show bcl-2 gene expression[34,35]. Our results are consistent with the report by Laporte *et al.,* who found the apoptosis to be considerably reduced in the basal layer of the epidermis in psoriasis, while being greatly enhanced at the site of regressive psoriatic plaque following PUVA therapy. They concluded that apoptosis was the key factor for post-therapeutic psoriasis regression, and used apoptotic index to calculate the time needed for psoriatic plaque regression after PUVA therapy, which was one to two months[42]. Yamamoto and Nishioka found increased bcl-2 expression in the basal layer of the epidermis in psoriatic skin following anthralin therapy[43]. Heenen *et al.* showed that, in addition to immunosuppressive action, methotrexate also influenced keratinocyte apoptosis[44]. Adisen *et al.* report on the same effects by use of calcipotriol and local steroids[45].

Accordingly, these literature reports and our own results suggest that the induction of keratinocyte apoptosis in the basal layer of the epidermis is one of the mechanisms of action exerted by antipsoriatic therapies currently in use. Our study confirmed the well-known efficacy of PUVA therapy and local corticosteroid therapy by comparison of pretherapeutic and post-therapeutic PASI score[4,46–48]. The statistically significant difference ($p<0.001$) in the PASI score between the experimental (PUVA treated) and control group of patients was not accidental, but resulted from the choice of therapeutic modality according to the dissemination of psoriatic lesions in particular patients (Table 1, Figure 1).

Conclusion

Study results demonstrated that both PUVA therapy and local corticosteroid therapy decreased the Ki-67 and F-8 expression, and increased bcl-2 expression. Thus, PUVA therapy and local corticosteroid therapy were confirmed to efficently act on the main pathogenetic mechanisms of psoriasis, i.e. to exert antiproliferative, antiangiogenic and apoptotic action. These very mechanisms appear to play a key role in therapeutic effects of most antipsoriatic treatments. PUVA resulted in excellent improvement of the clinical picture in psoriasis patients. In spite of the ever growing fear from UV exposure, even for therapeutic purpose, we believe that because of its efficacy PUVA will for a while remain the first choice therapy in the management of psoriasis vulgaris involving more than 30% of the skin area in patients free from contraindications for this therapy.

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ANTIPROLIFERACIJSKI, ANTIANGIOGENI I APOPTOTIČNI UČINAK FOTOKEMOTERAPIJE (PUVA) U BOLESNIKA S PSORIJAZOM

S A Ž E T A K

Cilj ovoga rada bio je istražiti antiproliferacijsko, antiangiogeno i apoptotično djelovanje fotokemoterapije (PUVA) i usporediti ga s kontrolnom skupinom bolesnika liječenih lokalnim steroidom. U istraživanje je bilo uključeno 60 bolesnika, od kojih je 30 liječeno PUVA terapijom, a 30 lokalnim steroidom. U istraživanju su primijenjene imunohistokemijske metode bojenja protutijelima Ki-67, F-8 i bcl-2. Pomoću anti protutijela Ki-67 mjeren je broj keratinocita u proliferaciji, pomoću anti protutijela F-8 prikazan je broj krvnih žila u dermisu, a pomoću protutijela bcl-2 prikazan je broj stanica koje pokazuju ekspresiju antiapoptotičkog onkoproteina bcl-2. Kod svih bolesnika je prije terapije i nakon šest tjedana određena vrijednost Ki-67, F-8 i bcl-2 te PUVA zbroj. Rezultati istraživanja pokazali su statistički značajne razlike u smanjenju broju keratinocita u proliferaciji u epidermisu ($p<0,001$) i smanjenju broja krvnih žila u dermisu ($p<0,001$) prije i nakon obiju vrsta liječenja. Vrijednost bcl-2 statistički je značajno porasla u bolesnika liječenih PUVA terapijom ($p=0,001$), kao i u kontrolnoj skupini, tj. dokazana je povećana apoptoza keratinocita nakon liječenja. Rezultati istraživanja dokazali su antiproliferacijsko, antiangiogeno i apoptotično djelovanje PUVA i lokalne steroidne terapije. Čini se kako su upravo istraživani mehanizmi djelovanja ključni u djelovanju većine antipsorijatičnih terapija.