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Expression of p53 Protein Before and After PUVA Treatment in Psoriasis

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We investigated the effect of the potentially carcinogenic psoralen plus UVA radiation (PUVA) therapy on the expression of p53 in skin of psoriatic patients. p53 antibodies DO7 and Pab240, antibodies against PCNA and Ki67 and the avidin-biotin immunoperoxidase complex method were used in the immunohistochemical staining of biopsy samples from non-lesional and lesional skin of 23 patients who received either trioxsalen bath PUVA or oral 8-methoxypsoralen PUVA. Biopsies were taken before and after a PUVA course. A modest expression of p53 was seen in psoriatic lesions in 17/ 21 patients before any treatment, probably as a physiological reaction to the hyperproliferation. Both p53 and the proliferation markers Ki67 and PCNA followed the same pattern, being more frequent in psoriatic lesions than in non-lesional skin. Exposure to PUVA induced an increase in p53 expression in non-lesional skin in 14/19 patients, putatively as a response to DNA damage caused by PUVA. In psoriatic lesions about half of the patients showed increased and half decreased expression of p53. The latter finding might be explained by decreased proliferation activity of the healing epidermis. In conclusion, p53 nuclear positivity in nonlesional skin after PUVA treatment is likely to be induced by DNA damage caused by PUVA, while in psoriatic lesions it could be a result of the combined effect of decreasing epidermal proliferation and DNA-damage. Key words: Ki67; PCNA; proliferation; skin; trioxsalen bath PUVA; oral 8-methoxypsoralen PUVA.

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Psoralen plus UVA (PUVA) photochemotherapy is frequently used in the treatment of psoriasis. PUVA mediates its effects largely through direct covalent binding of the psoralen to pyrimidine bases of the keratinocyte DNA under the influence of UVA irradiation. Normal DNA replication and cell division are disturbed and the hyperproliferation of the epidermis is reduced (1). At the same time the risk of DNA mutations increases. PUVA treatment causes transient systemic immunosuppression (2), which may promote the growth of skin cancer induced by PUVA or other carcinogenic agents. Both trioxsalen (TMP) and 8-methoxypsoralen (8-MOP) PUVA have been shown to be mutagenic in bacterial culture (3). Topical 8-MOP PUVA has been reported to cause tumours in mice, whereas TMP PUVA has not (4). Epidemiological follow-up studies in humans have shown a dose-dependent increase in the nonmelanoma skin cancer risk after systemic 8-MOP PUVA (5, 6), while such association has not been linked with TMP bath PUVA (7, 8.)

p53 is a transcription factor and an important regulator of the cell cycle (9). Wild-type p53 protein can inhibit cell division in response to DNA damage, allowing time for DNA repair. It can also trigger apoptosis. Inactivated p53 not only fails to carry out this activity but may also spur abnormal cell growth (10, 11). A mutation in the p53 gene has been found in most human tumour types and in approximately half of the nonmelanoma skin cancers (12). p53 immunoreactivity in PUVA tumours was found to be similar to that in other non-melanoma skin cancers in humans (13). Characteristic mutations in p53 at 5'TpG sites in squamous cell skin carcinomas of PUVA-treated psoriatic patients suggest that PUVA acts as a carcinogen (14). p53 immunohistochemistry has also been found to be positive in benign psoriasis lesions (15, 16). The origin of the p53 expression has been proposed to be the fast proliferation of the epidermis (15).

PCNA and Ki67 are nuclear proteins associated with cell cycle (17, 18). PCNA is also essential for DNA replication and excision repair (19). UV radiation has been shown to induce PCNA expression in epidermal cells in healthy human skin with a concurrent increase in p53 expression (20).

There is currently no data on the expression of p53 in nonlesional and lesional skin of psoriatic patients undergoing PUVA therapy, despite the evidence that the treatment may be carcinogenic. We have investigated how p53, Ki67 and PCNA are expressed in the skin of psoriatic patients before and after an ordinary PUVA course, either with TMP bath PUVA or oral 8-MOP PUVA.

MATERIAL AND METHODS

Patients

The study population comprised 7 female and 16 male patients of Caucasian origin (mean age 49 years, age range 24-73 years) with moderate to severe psoriasis. The patients received either TMP bath PUVA (n=18) or oral 8-MOP PUVA (n=5). Eight of the patients exposed to TMP bath PUVA were also treated with topical treatments including dithranol, corticosteroids and calcipotriol. Others did not get any topical treatments except emollients during the PUVA course. None of the patients had been exposed to artificial ultraviolet radiation within 15 days or systemic antipsoriatic medication within 2 months before admission. A written informed consent was obtained from all participants and the study was approved by the Ethics committees of the Medical Faculties in Oulu and Helsinki.

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PUVA treatment

TMP bath PUVA treatment was given once a day according to the usual regimen (8), with the exception that one forearm or the shoulders were left out of the bath and thus not presensitized with psoralen before the UVA irradiation. Briefly, 12.5-25 mg of TMP solution was mixed with approximately 150 l water. The patients bathed for 10 min, after which they were exposed to UVA light in a UVA cabin Waldmann UV 8001 K (Herbert Waldmann GmbH & Co., Villingen-Schwenningen, Germany) emitting approximately 11.0 mW/cm²of UVA at the treatment distance. The first UVA dose was 0.06 J/cm² and the dose was gradually increased. One treatment period included 6–15 treatments and the average cumulative UVA dose was 1.6 J/cm²(range 0.4–4.1 J/cm²).

Oral 8-MOP PUVA was given 3 times a week using PUVA cabins PUVA 2200 (Wolff System, Karatekniikka Oy, Finland) and PUVA 22A (Airam, Helsinki, Finland), emitting approximately 7.0 and 10.0 mW/cm²UVA, respectively. The initial UVA dose was 0.6 J/cm² and the dose was increased after every second treatment. The treatment period included 12-29 treatments and the average cumulative UVA dose was 56.3 J/cm²(range 29.7-112.8 J/cm²).

Skin biopsies

Two punch biopsies (diameter 4 mm) were taken under local anaesthesia before the first PUVA treatment: 1 from lesional and the other from adjacent non-lesional skin. Two biopsies were excised 24 h after the last treatment episode: 1 from lesional skin and 1 from non-lesional skin. One additional biopsy was taken from the patients who received TMP bath PUVA, from the extremity, which had been treated with UVA only. Biopsies were taken from the back, abdomen, chest, thigh, shin, upper and lower arm skin. Psoriatic lesions of 8 TMP bath PUVA patients were not completely cleared at the time of the latter biopsies. The biopsies were bisected, and half was snap frozen and stored at -70 °C, while the other half was fixed in 10% neutral formalin and embedded in paraffin blocks.

Immunohistochemical staining

The expression of p53, Ki67 and the proliferating cell nuclear antigen (PCNA) were demonstrated using the avidin – biotin complex immunoperoxidase method (Dakopatts, Copenhagen, Denmark) and the following antibodies: monoclonal antibody DO 7 (dilution 1:50) (Novo-castra Laboratories Ltd, Newcastle upon Tyne, UK) and murine monoclonal antibody PAb 240 (dilution 1:25) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) recognizing both wild- and mutant-type p53 proteins, monoclonal mouse primary antibody MIB-1 (Ki67) (Immunotech, Marseille, France) (dilution 1:25) and a murine monoclonal PCNA antibody (PC10, dilution 1:50) (DAKO A/S, Glostrup, Denmark).

Paraffin sections (4 µm) from each specimen were placed on poly-llysine (Sigma Chemicals, St Louis, MO, USA) coated glass slides and air-dried overnight at room temperature and stained within 2 weeks. The samples were de-waxed through xylene and absolute alcohol. Endogenous peroxidase activity was blocked by immersing the sections for 10 min in 0.1% hydrogen peroxide in absolute methanol and nonspecific binding was blocked by incubating the sections for 20 min in 20% foetal calf serum in phosphate buffered saline (PBS). Prior to incubation with DO7, Ki67 and PCNA antibodies, the sections were heated in 10 mM citric acid monohydrate (pH 6.0) in a microwave oven for 3 min. The DO7, Ki67 and PCNA antibodies were applied for 1 h at room temperature. The sections were then incubated with a secondary biotinylated anti-rabbit antibody (dilution 1: 400, 30 min) and the avidin-biotin complex (30 min) (both from Dakopatts, Copenhagen, Denmark). The colour was developed with diaminobenzidine, then the slides were lightly counterstained with haematoxylin. A lung carcinoma case strongly positive for p53by DO7 and a lymph node positive for PCNA and Ki67 were used as positive controls. Slides exposed to rabbit IgG (1:1000), non-immune rabbit serum (1:5) or PBS instead of the primary antibody served as negative controls.

Frozen sections of the same specimens were immunostained simi-

larly with a p53 antibody PAb 240. The incubation time for the antibody was 12-18 h at 4°C. PBS was used as a negative control and frozen sections of a lung carcinoma case strongly positive for p53 by PAb 240 as a positive control.

The immunostaining was repeated if the biopsy specimen had fallen off the glass slide or the staining was not satisfactory. In some cases all biopsy material was used up and the result could not be assessed. This happened randomly among the patients and only once for a patient treated with oral 8-MOP PUVA.

All the results were evaluated for the percentage of positive keratinocyte nuclei in the epidermis using an Olympus microscope BH-2 ($40 \times$ objective, diameter of field 400 µm). The examiner was not aware of the group the samples belonged to. At least 800 keratinocytes from 4-5 consecutive high-power fields were assessed. Cells with only granular cytoplasmic immunoreactivity were not taken into consideration.

Statistical analysis

Statistical analysis of the results was done using the Student's *t*-test for paired samples, linear regression analysis and Mantel-Haenszel chi-square test using SPSS.

RESULTS

Findings before PUVA treatment in non-lesional and lesional skin

p53. Approximately 0.3% (range 0-1.6%) of the keratinocytes in non-lesional skin and 1.4% (range 0-6.6%) in psoriatic lesions were immunopositive for DO7 before PUVA. A total of 17 out of 21 patients had higher count of DO7 positive keratinocytes in lesional skin than in non-lesional skin (95% confidence interval (CI) 0.4–1.8, p=0.003 in the *t*-test for paired samples). Two patients had lower and 2 an equal number of positive cells in lesional and non-lesional skin (Fig. 1). The results of 2 cases could not be assessed. DO7 positive keratinocytes were located predominantly in basal and suprabasal layers in the epidermis at all phases of the study. Positive staining was faint compared with the positive control, but clearly visible.

The immunohistochemical staining with PAb240 was negative both in lesional and non-lesional skin throughout the study. The age of the patients and duration of the psoriasis correlated poorly with the number of p53, Ki67 or PCNA positive cells in non-lesional and lesional skin before PUVA.

Ki67. An average of 5.8% of the keratinocytes in non-lesional skin and 16.6% in psoriatic lesions were immunopositive for Ki67 before any treatment. Eighteen out of 20 patients had higher count of Ki67 positive keratinocytes in lesional skin than in non-lesional skin (95% CI 4.8 to 16.8, p = 0.001 in the *t*-test for paired samples) (Fig. 1). The results of 3 cases could not be assessed.

PCNA. Approximately 14.5% of the keratinocytes in nonlesional skin and 48% in psoriatic lesions were immunopositive for PCNA before PUVA (Fig. 1). All patients had more PCNA positive keratinocytes in lesional skin than in non-lesional skin (95% CI 24 to 43, p = 0.00 in the *t*-test for paired samples).

The pattern seen with all the antibodies DO7, PCNA and Ki67 was very similar before PUVA. A total of 14 out of 17 patients showed a higher expression of both p53 and Ki67 in psoriatic lesions compared with non-lesional skin. A total of 17 out of 19 had a higher expression of both p53 and PCNA in psoriatic lesions compared with normal looking skin. We were, however, not able to demonstrate any linear correlation



Fig. 1. Percentage of p53 (DO7), Ki67 and PCNA positive keratinocytes in non-lesional skin and in psoriasis lesions before any treatment. Each line connects the samples of 1 patient.

between p53 and the proliferation markers (r = 0.1 for DO7/Ki67, r = 0.1 for DO7/PCNA in linear regression analysis).

Effect of PUVA on p53 and the proliferation markers in nonlesional and lesional skin

p53. The percentage of DO7 positive keratinocytes in nonlesional skin increased in 14/19 patients (from a mean of 0.3% to a mean of 2.2% of the keratinocytes), was unchanged in 1 patient and decreased in 4 patients after PUVA (from a mean of 0.3% to a mean of 0.03% of the keratinocytes) (95% CI 0.2–2.4, p = 0.02 in the *t*-test for paired samples). The same pattern was seen with both PUVA forms. The results of 4 cases could not be assessed.

The percentage of DO7 positive keratinocytes in psoriatic lesions increased in 8/19 patients (from 0.8% to 7.3%), remained the same in 2/19 patients and decreased in 9/19 patients after PUVA (from 2.3% to 0.9%). The results of 4 cases could not be evaluated. There were no great differences between the 2 PUVA forms.

Ki67. The percentage of Ki67 positive keratinocytes in nonlesional skin increased in 11/20 (from 3.2% to 6.9%) and decreased in 9 patients after PUVA (from 9.1% to 4.5%). Data could not be assessed in 3 cases.

The percentage of Ki67 positive keratinocytes in psoriatic lesions decreased in 15/22 (from 19% to13%) and increased in 7/22 patients after PUVA (from 8.9% to 23%) (p=0.9 in the *t*-test for paired samples). The samples of 1 patient could not be evaluated. The decrease in the keratinocyte counts seemed to be most pronounced in the group treated with oral 8-MOP PUVA.

PCNA. The percentage of PCNA positive keratinocytes in non-lesional skin increased in 11/23 patients (from 8.4% to 31%), decreased in 10/23 patients (from 19% to 6.9%) and remained unchanged in 2 cases after PUVA. This pattern was seen in the TMP bath PUVA group, while in the group treated with oral 8-MOP PUVA, the number of PCNA positive keratinocytes decreased in all 5 patients.

The percentage of PCNA positive keratinocytes in psoriatic lesions decreased in 15/23 (from 55% to 27%) and increased in 8/23 patients after PUVA (from 34% to 54%). The number of

immunopositive keratinocytes declined in 6/8 patients, who received TMP bath PUVA and topical treatments. There were no clear trends in the other groups.

No linear association could be demonstrated between p53 (DO7) and the proliferation markers in lesional or non-lesional skin after PUVA in the Mantel-Haenszel chi-square test (Table I).

The results in the TMP bath PUVA group with or without topical treatments were similar for p53 and Ki67.

Effect of UVA on p53 and proliferation markers in nonlesional skin

p53. The number of immunopositive keratinocytes increased in 9/15 patients (from 0.3% to 1.3%), was unchanged in 4/15 and decreased in 2 patients (from 0.5% to 0.1%) in non-lesional skin after only UVA irradiation. Data for 3 patients could not be assessed.

Ki67. The percentage of Ki67 positive keratinocytes in nonlesional skin decreased in 12/13 (from 7.7% to 3.7%) and increased in 1 patient after UVA irradiation (from 0.4% to 7.0%) (p = 0.05 in the *t*-test for paired samples). Results for 5 patients could not be evaluated.

PCNA. The percentage of PCNA positive keratinocytes in nonlesional skin increased in 7/12 (from 11% to 37%) and decreased in 5/12 patients after UVA irradiation (from 14%to 4.6%). The data of 6 patients could not be assessed.

In 5 patients an increase in p53 expression was seen both after UVA and PUVA. In 6/8 patients the expression of p53 and PCNA increased concomitantly after UVA. The expression of p53 increased while the number of Ki67 positive keratinocytes decreased in 6/8 patients after UVA. There was no linear association between p53 (DO7) and the proliferation markers in non-lesional skin after UVA estimated with Mantel-Haenszel chi-square test. (Table I)

DISCUSSION

Wild-type p53 is present in the nucleus of all normal mammalian cells. As a result of a short half-life (20-30 min) the protein concentration is usually below the detection level for

Table I. Change in the number of p53 (DO7), Ki67 and PCNA positive keratinocytes in non-lesional and lesional skin after PUVA or UVA, \uparrow increase, – unchanged, \downarrow decrease.

(a) Non-lesional skin after PUVA

p53	Ki67			PCNA		
	↑	-	\downarrow	↑	-	\downarrow
↑	8	_	5	7	_	7
\downarrow^-	- 1	_	-3	2	1 -	2

(b) Psoriasis lesion after PUVA

p53	Ki67			PCNA		
	↑	_	\downarrow	↑	_	\downarrow
↑	3	_	5	4	_	4
-	1	-	1	-	-	2
Ļ	2	-	6	2	-	7

5 pairs missing for p53/Ki67 and 4 pairs for p53/PCNA.

(c) Non-lesional skin after UVA

p53	Ki67			PCNA		
	↑	_	Ļ	\uparrow	_	\downarrow
↑	_	_	6	6	_	1
_	1	_	3	1	_	2
\downarrow	_	_	2	_	_	1

6 pairs missing for p53/Ki67 and 7 pairs for p53/PCNA.

immunohistochemical methods. Mutation in the p53 gene or interaction with viral or cellular proteins can stabilize the p53 protein leading to its immunohistochemical detection. Accumulation of p53 may also represent increased protein synthesis of wild-type p53 as a normal biological response to a higher frequency of DNA errors.

In this study we found an increased expression of p53 protein in both non-lesional and lesional skin of patients with psoriasis, who had not been exposed to artificial UV-radiation or topical treatments. Both p53 and the proliferation markers followed the same pattern, being more frequent in psoriatic lesions than in normal looking skin. A modest up-regulation of p53 has been demonstrated in psoriatic skin (15, 16) and it has been linked with hyperproliferation (15). Our study supports these findings. It is likely that the accumulation of p53 in the skin of psoriatic patients, seen prior to any treatment, was a physiological reaction of wild-type p53 trying to counteract the fast proliferation and to repair the possible DNA errors occurring in the rapidly proliferating tissue.

p53 mutations have been demonstrated in sun-exposed but histologically normal-looking epidermis in characteristic compact patches of p53 immunopositive keratinocytes. These mutations are likely to have little or no precancerous potential (21-23). Biopsy specimens obtained before PUVA course in this study revealed a dispersed pattern of p53 positive keratinocytes. Half of the samples from non-lesional skin adjacent to the psoriasis lesions were negative for DO7 and all samples from non-lesional and lesional skin were negative for Pab240. We did not perform any mutation analysis of p53 in this study but it is likely that the accumulated p53 before the PUVA course represented the wild-type form of the protein.

The number of p53 positive keratinocytes decreased in psoriatic lesions in about half of the patients after PUVA. Ki67 and PCNA expression decreased concurrently in about 65% of cases. The decrease in p53 expression in psoriatic lesions could, therefore, be secondary to decreased proliferation activity of the healing epidermis advocating the hypothesis above. We were, however, unable to demonstrate any clear association between proliferation marker activity and p53 expression in individual cases.

The number of p53 positive keratinocytes increased in half of the patients in psoriatic lesions and in 75% of the non-lesional skin samples after PUVA. There were no clear trends in the expression of the proliferation markers. p53 response in nonlesional skin was likely to occur as a response to DNA damage caused by PUVA. PUVA is known to be mutagenic and carcinogenic (1, 3) and also UVA alone can cause DNA damage in human epidermis (24). UVA irradiation has been shown to induce p53 response in normal human skin due to a transient post-translational stabilization of the protein in response to either direct DNA damage or the ability of the cell to detect DNA damage (25). The pattern of p53 expression was similar after both oral 8-MOP PUVA and TMP bath PUVA. These 2 PUVA forms have a similar mechanism of action at the DNA level, but UVA doses needed in 8-MOP PUVA are about 15 times higher than in TMP bath PUVA, due to the greater phototoxicity of TMP.

In conclusion, p53 nuclear positivity in non-lesional skin after PUVA treatment was likely to be induced by DNA damage caused by PUVA, while in psoriatic lesions it was putatively a result of the combined effect of decreasing epidermal proliferation and DNA damage.

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