

## Influence of Activation and Differentiation of Cells on the Effectiveness of Photodynamic Therapy

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**Photodynamic therapy combines photosensitizers absorbing light in the red spectral region and irradiation with light of the corresponding wavelength. To analyse the influence of cell differentiation on susceptibility to photodynamic therapy, we compared the proliferation inhibition induced by photodynamic therapy on normal human keratinocytes, spontaneously transformed human keratinocyte cell line HaCat and on squamous cell carcinoma lines. Cells were irradiated with polychromatic light in the presence of the photosensitizers Photosan-3 and methylene blue as well as the precursor of protoporphyrin, 5-aminolevulinic acid. When incubated with Photosan-3, normal human keratinocytes exhibited an ED<sub>50</sub> about 10-fold lower than the other cell lines studied. When methylene blue and 5-aminolevulinic acid were used, photodynamic therapy had comparable effects on all cell types. Stimulation of normal human keratinocytes with either EGF-alpha or IFN-gamma resulted in an increased susceptibility to photodynamic therapy when 5-aminolevulinic acid was used. This effect was more pronounced in the case of EGF-alpha. Our experiments suggest that activation and differentiation are two important parameters determining susceptibility to photodynamic therapy.**  
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Photodynamic therapy (PDT) involves the combined use of photosensitizing drugs absorbing light in the visible spectral region, e.g. haematoporphyrin derivatives, and irradiation with light of the corresponding wavelength for the treatment of malignant and benign diseases. In dermatology treatment of keratinocyte-derived tumours by PDT has been extensively studied (1–3).

Photoactivation of the photosensitizing drugs, which selectively accumulate in malignant tissues (4, 5), leads to the formation of cytotoxic molecules (e.g. singlet oxygen) and causes tumour destruction with less damage to surrounding normal tissue (3). However, recent data suggest the possibility of successful application of PDT in disseminated dermatoses, e.g. cutaneous lymphoma and psoriasis (6–8). Moreover, it has been demonstrated that light of 600–700 nm wavelength emitted by a bulb is as potent as monochromatic light in inducing photodynamic reactions (9). Nevertheless, at present very limited data are available concerning parameters influencing target susceptibility to PDT. In order to further explore parameters influencing the effectiveness of PDT, we investigated the effect of differentiation and activation of cells on susceptibility to PDT.

A comparison was made between the proliferation inhibition induced by PDT on normal human keratinocytes (KCs), spontaneously transformed human keratinocyte cell line HaCat and on

squamous cell carcinoma lines SCL1 and SCL2. Furthermore we studied the influence of stimulation with either IFN-gamma known to induce KC differentiation (10) or EGF-alpha, a cytokine controlling and stimulating the proliferation of epidermal and epithelial cells (11).

### MATERIAL AND METHODS

#### *Cell lines*

The cell lines SCL1 and SCL2, derived from squamous cell carcinomas of the skin, and the cell line HaCat, derived from spontaneously immortalized human keratinocytes, were provided by Prof. N. E. Fusenig (DKFZ, Heidelberg, Germany) (12, 13). Native human keratinocytes were either purchased from PromoCell (Heidelberg, Germany), as primary cells or established using human adult skin from the trunk by incubation with trypsin (at 4° for 24 h) and subsequent cultivation, as described elsewhere (14).

#### *Photosensitizers*

Photosan-3 (PS), a hematoporphyrin derivative similar to Photofrin II, was purchased from Seelab (Germany). PS is a mixture of different porphyrins, including hematoporphyrin IX, protoporphyrin IX, deuteroporphyrin IX and dihematoporphyrinether. In aqueous solution, these porphyrins are present as monomers, dimers, or higher aggregates. Five-aminolevulinic acid (ALA) was obtained from Merck (Germany). This substance, once taken up by cells, is metabolized into protoporphyrin IX which represents the photosensitizing agent. ALA was diluted in medium and neutralized to pH 7.2 with NaOH prior to further use. It was used immediately afterwards. Methylene blue (MB) was purchased from Merck. All photosensitizers were diluted in medium before they were added to cell cultures.

#### *Light source*

Irradiation was performed using a polychromatic light source (PDT 1200, Waldmann, Germany). Light emitted by this metal-halogen-ARC lamp was filtered, so that only light of 600–700 nm wavelength would pass the filter. The spectral output of the lamp is about constant throughout this range. The power density was set at 100 mW/cm<sup>2</sup>.

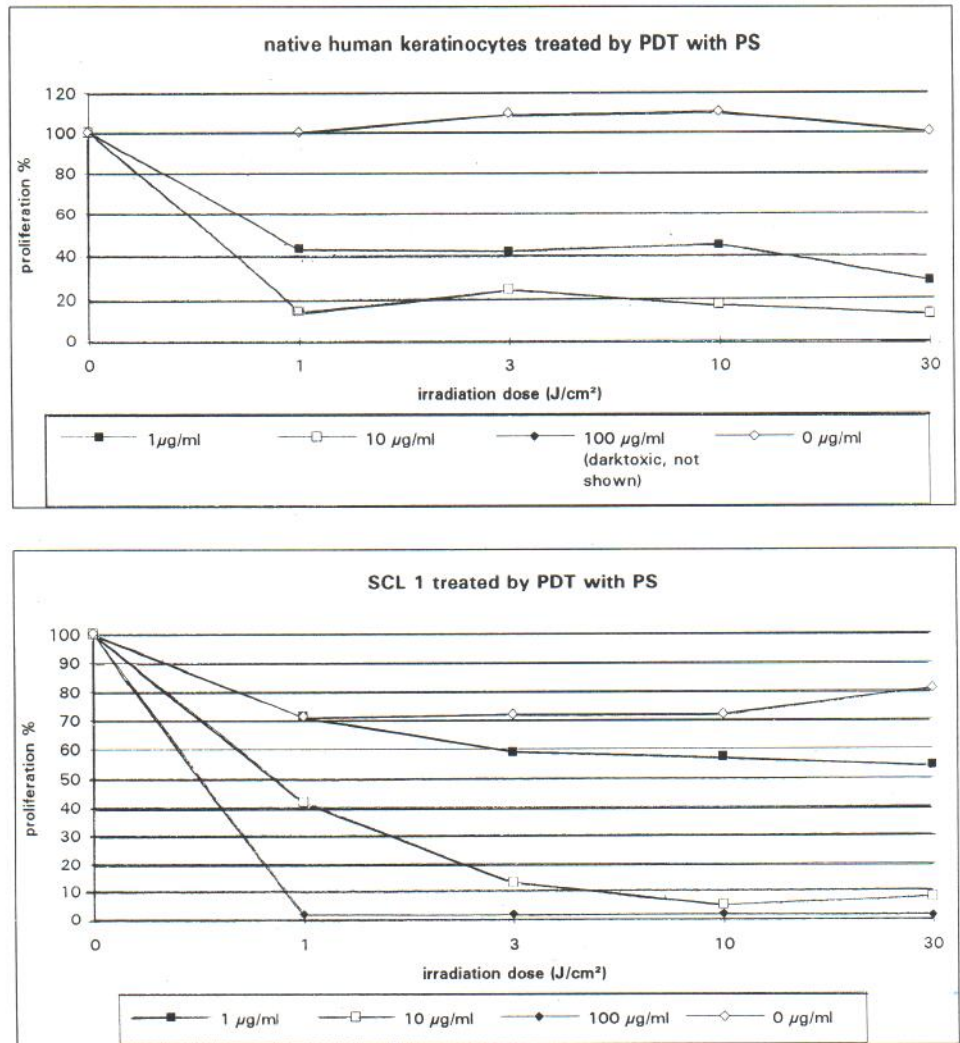
#### *Irradiation protocol*

Irradiation was performed as previously described (8). Briefly, to determine the effect of PDT on proliferation, we placed  $3 \times 10^3$  to  $10 \times 10^3$  cells per well in flat bottom 96 well microtiter plates. The photosensitizers were added to final concentrations of 30 µg/ml to 300 µg/ml (ALA), 1 µg/ml to 100 µg/ml (PS), 150 ng/ml to 15 µg/ml (MB). The plates were wrapped in aluminum foil and kept in the dark to prevent uncontrolled light exposure. After 2 h incubation with the sensitizers, cells were washed once with medium prior to and after irradiation with polychromatic light in doses of 1 J/cm<sup>2</sup> to 30 J/cm<sup>2</sup>. As controls, cell cultures not incubated with any photosensitizer were treated in the same way. After irradiation <sup>3</sup>H-thymidine was added and the uptake was determined 12 h later. Experiments were done in triplicates. Proliferation of cells in the presence of the photosensitizer was defined as 100%. We subsequently determined the dose yielding a 50% decrease in cell proliferation (= ED<sub>50</sub>).

In order for us to confirm the results obtained by <sup>3</sup>H-thymidine uptake, proliferation was also determined by total cell counting.



Fig. 1. Dose-response curves of the cell line SCL 1 and native human keratinocytes treated by PDT with Photosan-3 (PS), using a polychromatic light source.



#### Cytokine stimulation

For stimulation cells were incubated 48 h prior to PDT treatment with either 1000 U/ml IFN-gamma (Thomae, Germany) or 10 ng/ml EGF-alpha (Hermann Biermann, Germany). These concentrations were determined by titration screening for induction of HLA class II expression and proliferation, respectively.

## RESULTS

#### Influence of differentiation on susceptibility to PDT

No differences in susceptibility to PDT could be observed among the different keratinocyte types when ALA and MB were used: In the cell lines HaCat, SCL1 and SCL2, light doses higher than 30 J/cm<sup>2</sup> had to be applied to yield a 50% inhibition of <sup>3</sup>H-thymidine uptake in the presence of 300 µg/ml ALA, a concentration not leading to darktoxic effects. The same was true for normal human keratinocytes. When MB was used, the different cell lines and the KCs again responded, similar to PDT, with corresponding concentrations of MB (data not shown).

However, and this is in contrast to the other sensitizers, when PS was applied as photosensitizer normal keratinocytes showed the highest response; when concentrations of 1 µg/ml PS were used the cell lines SCL1, SCL2 and HaCat had to be treated with

light doses higher than 30 J/cm<sup>2</sup> to achieve 50% inhibition of <sup>3</sup>H-thymidine uptake. However, normal human keratinocytes exhibited a clearly lower ED<sub>50</sub> (< 1 J/cm<sup>2</sup>), when treated in the same way (Fig. 1).

Total cell counts of HaCat and KCs 24 h after treatment by PDT using PS and MB confirmed the results obtained by <sup>3</sup>H-thymidine uptake. When cell number was used as read-out, the data obtained by <sup>3</sup>H-thymidine-uptake were confirmed.

#### Influence of IFN-gamma on susceptibility to PDT

There were no differences in effectiveness of PDT towards keratinocytes following stimulation by INF-gamma, compared to non-stimulated ones, when PS and MB were used. Similar ED<sub>50</sub> was obtained in stimulated as well as non-stimulated keratinocyte types. However, when ALA was used the ED<sub>50</sub> for normal human keratinocytes stimulated by IFN-gamma was found to be considerably lower. The ED<sub>50</sub> value was 19 J/cm<sup>2</sup> for stimulated KCs, whereas non-stimulated KCs had to be treated with light doses higher than 30 J/cm<sup>2</sup> when incubated with 150 µg/ml ALA. In the cell lines HaCat, SCL1 and SCL2 no differences were observed between non-stimulated and stimulated cells when ALA was used (data not shown).



Table 1.  $ED_{50}$  ( $J/cm^2$ ) of cell lines HaCat (HaC), SCL1, SCL2 and normal human keratinocytes (KCs) in the presence of the photosensitizer Photosan-3 (PS), using polychromatic light (standard deviation was always <15%)

	PDT using PS															
	KCs				HaC				SCL1				SCL2			
	without stim.				stim. with INF-gamma				stim. with EGF-alpha							
1 $\mu g/ml$	<1	>30	>30	>30	2	>30	>30	>30	4	>30	>30	>30				
10 $\mu g/ml$	<1	3	<1	2	<1	2	<1	3	<1	<1	<1	4				
100 $\mu g/ml$	tox.	<1	<1	<1	tox.	<1	<1	<1	tox.	<1	<1	<1				

#### Influence of EGF-alpha on susceptibility to PDT

Stimulation by EGF-alpha led to similar results as stimulation by INF-gamma. Again, when PS and MB were used no differences were seen between non-stimulated and stimulated cells. However, in normal human KCs a clearly lower  $ED_{50}$  was found, compared to non-stimulated normal KCs when ALA was used. This effect was more pronounced than that obtained with INF-gamma and was restricted to normal human KCs, whereas the cell lines HaCat, SCL1 and SCL2 did not show any other responses when stimulated with EGF-alpha (data not shown).

These results were confirmed using total cell counts as read-out.

#### DISCUSSION

In the present study we investigated the inhibitory effect of PDT on proliferation of different types of keratinocytes. The keratinocyte cell lines HaCat (immortalized keratinocytes), SCL1, SCL2 (squamous cell carcinoma lines) and normal human keratinocytes were incubated with the PS, MB and ALA. SCL1 and SCL2 are lines established from squamous cell carcinomas and thus presumably represent a differentiation state different from native KCs, whereas HaCat cells exhibit several features of normal KCs. Thus, using these lines as well as native human keratinocytes, we studied the influence of differentiation on the effects of PDT.

When MB and ALA were used, no profound differences in phototoxicity were found in the different keratinocyte cell types. In the case of PS the normal keratinocytes were more sensitive to PDT than the cell lines SCL1, SCL2 and HaCat.

The unexpectedly high response of normal KCs to PDT with PS favours topical application of this sensitizer. In the literature, following systemic application of PS, necrosis of irradiated peritumoral normal skin at light doses applied for tumour eradication is reported (15).

When cells were stimulated by INF-gamma, photodynamic activity was enhanced in the case of normal human KCs when ALA was applied but not when the other sensitizers were used. The susceptibility of the cell lines HaCat, SCL1 and SCL2 was not altered when stimulated by INF-gamma. Similar results – an even more pronounced susceptibility of normal human KCs in the case of ALA – were obtained when EGF-alpha was used for stimulation. Our results suggest that activation and differentiation are both important parameters influencing susceptibility to

PDT. However, this seems to be true only for certain sensitizers, e.g. porphyrins, since susceptibility of cells incubated with MB could not be altered by either activation or differentiation.

Whether this indicates different photodynamic mechanisms is to be further investigated. In fact, fluorescence analysis concerning the location of different sensitizers within cell structures revealed striking differences between the sensitizers MB and PS (8).

Differentiation was the main important factor influencing susceptibility of cells to PDT with PS, whereas activation of cells seemed to be of main importance when ALA was used. This contrast might be explained by the differences in metabolism of ALA and PS: PS acts directly as photosensitizing drug whereas ALA has to be metabolized by the cells into protoporphyrin IX, which represents the photosensitizing agent (16). It seems possible that activated cells might have either an increased uptake of ALA or an increased synthesis of protoporphyrin IX and therefore respond more pronouncedly to PDT than non-activated cells. KC stimulation with either INF-gamma or EGF-alpha results in a higher susceptibility to PDT when ALA is used. Thus, PDT should be particularly useful for the treatment of diseases characterized by KC hyperproliferation caused by these cytokines.

For example hyperproliferative diseases of the epidermal keratinocytes, as psoriasis vulgaris, are characterized by overexpression and altered distribution of the epidermal growth factor (transforming growth factor) – alpha receptor and of TGF-alpha, a peptide factor homologous to EGF-alpha (17), as well as psoriasis by the presence of INF-gamma (18). In fact, our recently published initial observations support the hypothesis that topical polychromatic PDT is a safe and effective treatment modality in psoriasis (6, 9). If proliferation inhibition of keratinocytes is a relevant mechanism of PDT in vivo it might be responsible – besides reduced cytokine production (9) – for the effectiveness of PDT in psoriasis and could also be useful in other dermatoses associated with epidermal hyperplasia.

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