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INVESTIGATIVE REPORT

In vivo Porphyrin Production by *P. acnes* in Untreated Acne Patients and its Modulation by Acne Treatment

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***Propionibacterium acnes* is often discussed as a contributing pathogenic factor in the aetiology of acne lesions. The aim of this study was to test which porphyrin patterns are synthesized by *P. acnes in vivo* in untreated acne patients and during standard acne regimens. These photosensitive compounds are potential targets for photodynamic therapy of acne and need to be better characterized in the skin. Using high-performance liquid chromatography coproporphyrin III was the main porphyrin identified in all patients. Coproporphyrin I and protoporphyrin were found at considerably lower concentrations. When the porphyrin concentration of individual patients receiving isotretinoin was analysed repeatedly over time, clinical improvement was associated with lowered levels of porphyrins. Statistical analysis demonstrated a significant reduction in the porphyrin fractions only in the isotretinoin group which was associated with clinical improvement 2 months after starting therapy.**

Key words: Propionibacterium acnes; coproporphyrin I; coproporphyrin III; protoporphyrin.

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Acne is a chronic inflammatory disorder of the pilosebaceous follicles with a multifactorial aetiology. Androgen-induced seborrhoea, follicular hyperkeratinization, and the commensal bacterium *Propionibacterium acnes* appear to play a role in its pathophysiology (1). *P. acnes* not only colonizes sebaceous follicles, but there are also some abnormalities of the growth or metabolism of this organism in acne resulting in comedogenesis and the development of inflammatory acne lesions (2). In recently published papers the co-incubation of keratinocytes with viable, but not heat-killed, *P. acnes* modulated the cytokine response for interleukins (IL)-1α and IL-1β, tumour necrosis factor, granulocyte monocyte colony stimulating factor, and IL-8 (3–5), while other studies failed to demonstrate such cytokine modulations by *P. acnes* (6–8).

Intradermal injection of *P. acnes* into the ears of rats produced chronic inflammation with formation of acneiform lesions (9). After injecting bacterial suspensions into sterile keratin cysts, an inflammation was detected morphologically (10). The identity of these inflammatory factors has not been clarified conclusively, but various enzymes produced by *P. acnes*, such as lipases, hyaluronidases, proteinases, toxins and microbial allergens are suspected (11).

Porphyrins are further metabolic products of *Propionibacteria.* Their existence has been proved by coral-red fluorescence in the follicle openings by examining facial skin under Wood's light (ultraviolet-A 320–400 nm) (12). Porphyrins might contribute to the perifollicular inflammatory reaction by their cytotoxic effect and by stimulating expression of keratinocyte-derived IL-8 (5). After rupture of the follicle epithelia, porphyrins secreted perifollicularly could also contribute to the inflammatory reaction of the follicle or its environment by favouring the development of cytotoxic substances such as squalene peroxide possibly via singlet oxygen (13).

Successful therapy of acne with visible light by using a blue-light high-energy lamp was first reported by Meffert et al. (14). Recently, improvement of acne was reported after blue light-mediated photo-inactivation of *P. acnes* by its endogenic porphyrins (15, 16). While the *in vitro* porphyrin pattern of *P. acnes* has been qualitatively and quantitatively analysed in several studies by high-performance liquid chromatography (HPLC) (15, 17, 18) there is still a lack of *in vivo* HPLC investigations. In addition, the effect of isotretinoin as the most effective acne therapy on the *in vivo* porphyrin pattern remains to be investigated.

The aim of this study was to analyse both qualitatively and quantitatively using a highly sensitive HPLC, the porphyrins produced by *P. acnes in vivo* before and after oral isotretinoin therapy, in comparison with other acne treatments.

MATERIALS AND METHODS

Patients and collection of samples

Fifty-eight patients (32 women and 26 men) with acne papulopustulosa and conglobata were examined in a randomized trial

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after evaluation with a standardized questionnaire. Studies were carried out following informed consent and in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 1983. The patients' age ranged from 13 to 37 years, with a median of 20 years. Porphyrins were measured in skin samples, both in 16 patients before treatment and another 16 patients 2 months after starting oral isotretinoin therapy with doses ranging from 0.2 mg/kg/day to 0.5 mg/kg/day. In 9 additional patients the porphyrin levels were analysed intra-individually before isotretinoin treatment and 2 months after having started therapy. Also investigated were samples of 17 patients receiving oral minocycline 50–100 mg/day combined with topical benzoyl peroxide for 2 months. In all treated groups the samples were taken 2 months after having started acne therapy.

After cleansing of the acne lesions with 70% ethanol the contents of 3 lesions were squeezed out and collected using a comedo extractor. The weight of the samples was measured by high-precision laboratory scale (Sartorius, Goettingen, Germany).

Microbiological analysis

Part of the material described above was used to isolate *Propionibacterium spp.* under micro-aerophilic and dark conditions on brain-heart infusion agar at pH 7.1 and 37°C. The jars contained a mixture of hydrogen and carbon dioxide (95:5, v/v) and the oxygen content was less than 5%. Using the biochemical identification system API 20 A (BioMérieux, Lyon, France), all *P. acnes* isolates could be characterized as *P. acnes* serotype I.

Qualitative and quantitative porphyrin analysis

The samples were suspended in 2 ml 0.9% NaCl solution and homogenized in a mortar. The homogenizate was transferred to Eppendorf tubes (Eppendorf, Hamburg, Germany) washed with 1 ml 0.9% NaCl solution and centrifuged for 4 min at 2000 rpm in a 5415°C Eppendorf centrifuge. The residue was disposed of and 50 µl Celite® (Fluka, Paesel, Germany), a filtration aid consisting of kieselguhr of varied granular size and 250 µl ethylacetate/ethanoic acid (4:1) were added. Finally, the suspension was mixed for 10 sec in a vortexer and then centrifuged for 1 min at 1500 rpm. The residue was transferred via a pipette into another Eppendorf tube and 250 µl 1.5 M hydrochloric acid (HCl) was added. The solution was mixed for 10 sec in the vortexer. Finally, approximately 300 µl of the lower phase, which contains the porphyrins, was transferred via a pipette to another Eppendorf tube.

Because of the high photosensitivity of porphyrins, the extracted samples were kept in the dark at 4°C and then reprocessed for HPLC after adding HCl not more than 2 h later. Prior to HPLC analysis the porphyrin containing extract was mixed with 2 ng mesoporphyrin as an internal standard (SIGMA, Deisenhofen, Germany) and loaded onto a C18(e) solid phase extraction column (Separtis, Grenzach-Wyhlen, Germany). After washing with water, the porphyrins were eluted with acetone/methanol (1:1) containing 1% triethylamine; the content of specific porphyrins was quantified as described by Jacob & Luppa (19). The amounts of porphyrins analysed were related to the weight of comedones processed (ng porphyrin/mg tissue).

Statistical analysis

The results were evaluated statistically with the "SPSS 10.0 for Windows" statistics program. The relationship between porphyrin concentrations from untreated patients and after therapy was tested using the Mann-Whitney U test, a non-parametric test for unassociated random samples. A p value of 0.05 or less was considered significant.

RESULTS

Microbiological analysis

P. acnes (90%) was the predominant *Propionibacterium spp.* isolated from the acne lesions. We could only very rarely isolate *P. granulosum* (8%) or *P. avidum* (2%). Comparative analysis of *in vivo* porphyrin patterns produced by *P. acnes* and *P. granulosum* revealed no differences (not shown). We therefore decided to focus our studies on *P. acnes*.

Qualitative and quantitative analysis of porphyrins before and after acne therapy

Qualitative HPLC analysis demonstrated a uniform porphyrin profile in all investigated patient samples from comedones or inflammatory acne lesions (results not shown). In each case, coproporphyrin III accounted for the largest fraction. Coproporphyrin I and protoporphyrin were present in lower concentrations, whereas polar porphyrins were not detected. In general, the amount of coproporphyrin III correlated highly (correlation coefficient = 0.952) with the number of colony forming units of *Propionibacteria* isolated from the blackheads (Fig. 1).

In contrast to the group of untreated control patients the coproporphyrin I, III and protoporphyrin values for patients treated with isotretinoin for 2 months were significantly lower (Table I).

In 9 patients porphyrins were analysed during the course of isotretinoin treatment. Six of them also showed decreased porphyrin values 2 months after starting isotretinoin therapy, which correlated clinically with the improvement of the skin conditions. Interestingly in 3 patients with unimproved acne lesion the coproporphyrin levels were unchanged or increasing (Fig. 2).

Coproporphyrin III values for patients treated with oral minocycline plus topical benzoyl peroxide for 2

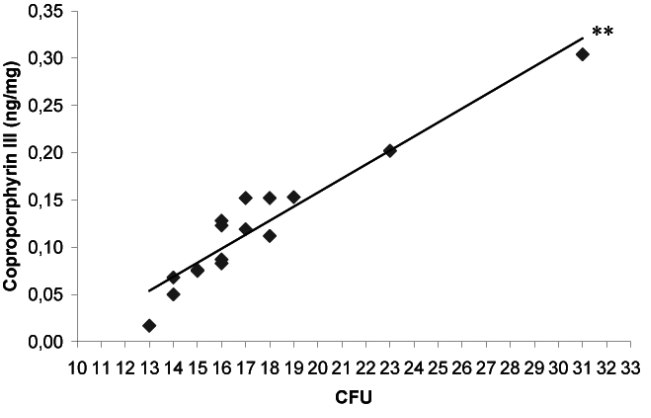

Fig. 1. Regression analysis demonstrating a highly significant correlation (**$p < 0.001$, correlation coefficient = 0.952) between colony forming units (CFU) of *P. acnes* and the sum of coproporphyrin III levels isolated from 3 lesions.

Table I. *Mean ± SD porphyrin concentrations (ng/mg) in skin extracts from untreated acne patients and patients treated for 2 months with isotretinoin or minocycline/benzoyl peroxide.*

	Untreated	Isotretinoin	Minocycline/ Benzoyl peroxide
	(n = 16)	(n = 16)	(n = 17)
Coproporphyrin III	0.119 ± 0.017	0.075 ± 0.024**	0.149 ± 0.034
Coproporphyrin I	0.012 ± 0.002	0.007 ± 0.002*	0.010 ± 0.002
Protoporphyrin	0.024 ± 0.007	0.006 ± 0.002*	0.007 ± 0.002

Statistically significant differences (*p < 0.05) in coproporphyrin I (*p = 0.035), coproporphyrin III (*p = 0.008) and protoporphyrin levels (*p = 0.031) between untreated and isotretinoin treated patients.

months were insignificantly increased compared with untreated patients, while coproporphyrin I and protoporphyrin levels were slightly decreased (see Table I).

DISCUSSION

The porphyrins in the present study were separated by HPLC. The advantage of this technique over other chromatographic methods lies in the high-resolution power, enabling separation of porphyrin isomers even at the picogram level. Mesoporphyrin was added to the samples as internal standard. The mean mesoporphyrin recovery rate of 97% showed that only non-significant amounts of porphyrin were lost during sample preparation.

In the present study *P. acnes* (90%) was the predominant *Propionibacterium spp.* isolated from the acne lesions, while *P. granulosum* (8%) or *P. avidum* (2%) were only rarely isolated. As comparative porphyrin analyses were similar in all investigated samples, it is tempting to speculate that porphyrins are *in vivo* mainly produced by *P. acnes*. Contamination of our *in vivo* samples with keratinocyte-derived coproporphyrin III can be excluded, as microspectrofluorometric emission spectra has failed to demonstrate this porphyrin fraction in diseased as well as in normal skin (20).

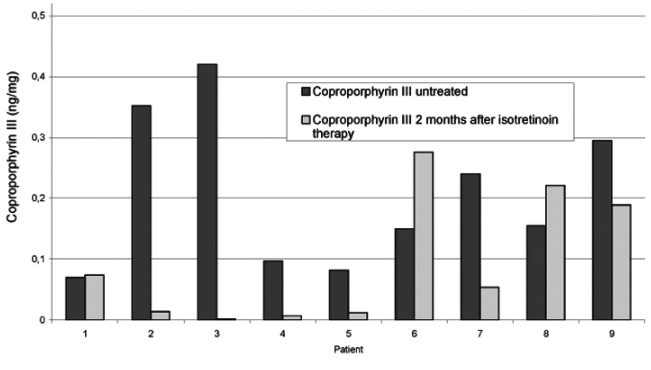

Fig. 2. Comparison of coproporphyrin III concentration in individual patients before therapy and 2 months after starting oral isotretinoin therapy.

HPLC analysis of acne extractions revealed a qualitatively uniform porphyrin profile, with coproporphyrin III as the major porphyrin fraction, whereas coproporphyrin I was present in low concentrations, and protoporphyrin hardly at all. This result matches the porphyrin formation of previously published *in vivo* (12, 21, 22) and *in vitro* studies (23–25).

The considerable inter-individual variations in the porphyrin concentration observed in the present study have also been seen in previous investigations (21, 23, 24). Similar porphyrin patterns in comedones and inflammatory acne lesions implicate that inflammation is not caused by modulation of the porphyrin profile.

A collective comparison of the porphyrin composition in untreated patients and patients treated with isotretinoin or minocycline plus benzoyl peroxide revealed no qualitative differences. These results suggest that the porphyrin profile secreted by *P. acnes* is not modulated by these standard therapies. Concentrations of all three porphyrin fractions (coproporphyrin I, coproporphyrin III, protoporphyrin), however, were significantly reduced in the isotretinoin treated group compared with an untreated group of acne patients. The reduction in porphyrin values was confirmed in repeated, vertical measurements in single patients before and 2 months after having started isotretinoin therapy and correlated with an improvement of acne. In contrast, in patients receiving oral minocycline therapy in combination with topical benzoyl peroxide, HPLC analyses demonstrated no reduction in porphyrin concentrations. Inadequate patient compliance and an increasing tendency towards resistance to minocycline of *P. acnes* are two possible reasons for this finding (26).

The association of clinical improvement with a significant reduction in the porphyrin concentration implicates a decreased number of *P. acnes* as a result of isotretinoin therapy, and this has also been shown repeatedly by others. In corollary, we suggest that oral isotretinoin is more effective in reducing *P. acnes* and suppressing porphyrin production than oral minocycline/topical benzoyl peroxide therapy. The role of porphyrins as a factor contributing to the pathogenesis of acne is still unclear. Coproporphyrin III can induce a keratinocytic IL-8 response and might via this mechanism contribute to the development of inflammation (5). Knowledge of the relatively large amounts of porphyrins in acne skin and the identification of the secretion pattern of porphyrins by *P. acnes in vivo* might be helpful to improve the efficacy of photodynamic destruction of these bacteria (27). Treating acne patients with phototherapy is possible without externally applied photosensitizers because of the presence of endogenous porphyrins. This modality is receiving increased attention because of the increasing tendency for antibiotic resistance of *P. acnes* (26). Initial studies with blue light reported encouraging results (15, 16, 28).

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