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The Study of Endogenous Porphyrins in Human Skin and Their Potential for Photodynamic Therapy by Laser Induced Fluorescence Spectroscopy

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Abstract. Human skin shows a strong autofluorescence in the red spectral region when excited by the 407 nm radiation of a krypton ion laser. The spectrum consists of three main peaks around 600, 620 and 640 nm, which are typical for metalloporphyrins such as Zn–protoporphyrin, coproporphyrin and free protoporphyrin IX, and perhaps represent a mixture of these compounds. The fluorescence is located in sebaceous follicles which contain large amounts of the porphyrin-producing skin bacterium Propionibacterium acnes. Irradiation, especially with violet light, reduces both the integral fluorescence intensity and the number of living bacteria. The process of photobleaching is oxygen-dependent. In addition, irradiation results in the formation of fluorescent photoproducts with spectral bands similar to photo-protoporphyrin. It seems to be possible to use the endogenous porphyrins for a photodynamic therapy of acne vulgaris and to monitor the therapeutic effect by the simultaneous measurement of spectral changes.

INTRODUCTION

Ultra-violet radiation induces luminescence of the human skin in the red spectral region (1) due to the lipophile microorganism Propionibacterium acnes (2). This bacterium belongs to the normal microbial flora of the skin and is involved in the pathogenesis of the disease acne vulgaris. This disease is connected with a hyperkeratosis of the pilosebaceous follicles in addition to an increased sebum production. This results in the development of comedones, which can induce inflammation. The Propionibacterium acnes promotes the inflammatory reactions (3–6).

Propionibacterium acnes is able to produce fluorescent porphyrins. Whereas haem is non-fluorescent (or its fluorescence intensity is extremely low), the intermediate products of haem synthesis such as protoporphyrin, uroporphyrin, and coproporphyrin are fluorescent metal-free porphyrins. Low iron concentration and defects of the pyrrole synthesis can cause a high accumulation of these porphyrins which leads, for example, to the disease porphyria. In the Propionibacterium acnes significant amounts of coproporphyrin III and protoporphyrin IX have been found (7–9).

In contrast to the use of exogeneous porphyrins in the photodynamic therapy of tumours (10), the intrinsic production of porphyrins by Propionibacterium acnes may possibly be used for the treatment of acne vulgaris with visible light. Meffert et al (11) has reported the treatment of 34 patients with acne vulgaris by means of blue light.

Relatively few studies have been reported on the effect of visible light on Propionibacterium acnes, e.g. Meffert et al (11), Kjeldstad (12) and Kjeldstad and Johnsson (13). The aim of this paper is to report on the fluorescence behaviour during irradiation of these bacteria in cultured colonies as well as in the microbial flora of the human skin and in comedones.

MATERIALS AND METHODS

Spectrometer

The apparatus for laser-induced in vivo fluorescence spectrometry is shown in Fig. 1.

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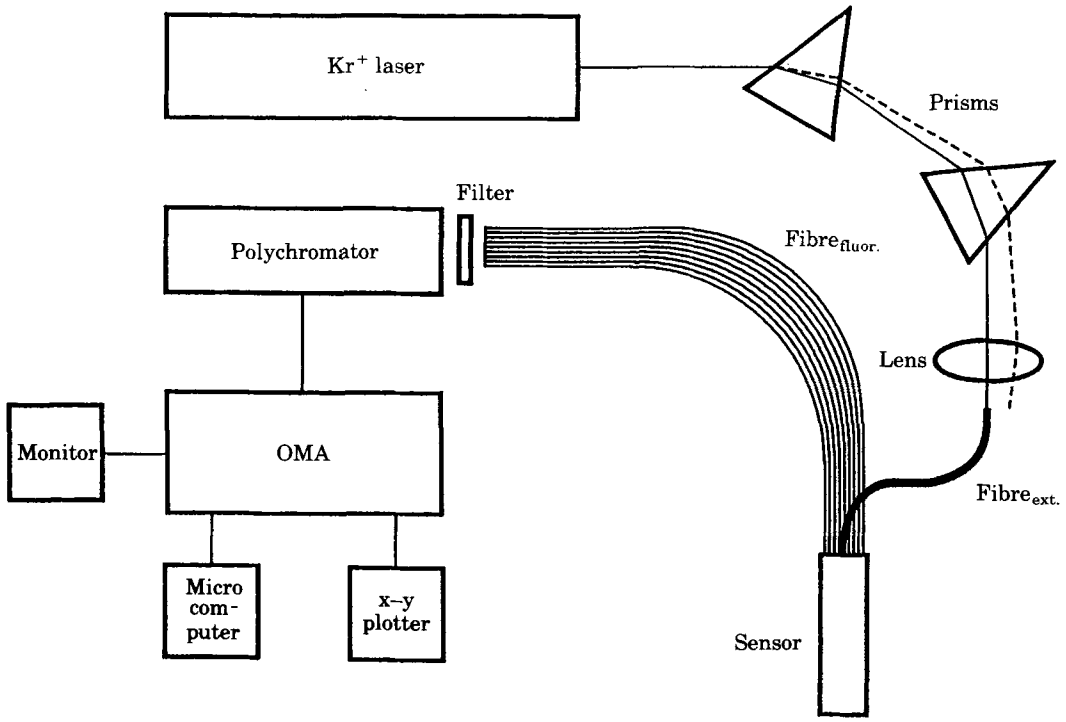

Fig. 1. Apparatus for the fluorescence detection. The diffraction prisms discriminate the background laser radiation. The filter in front of the entrance slit of the polychromator reflects the scattered excitation light. The distance between the excitation fibre and the bundle of detection fibres is variable.

Fluorescence was excited by the 406.7 nm, the 415.4 nm or the 356.4 nm u.v. line of a krypton ion laser coupled to a 600 μm quartz fibre. The spontaneous emission of the laser and the luminescence of the Brewster windows are separated from the excitation line by the use of two diffraction prisms (14, 15). The fluorescence light was collected by a polished quartz fibre bundle which was arranged around the excitation fibre. The end of the bundle was adapted to the input slit of a polychromator. A dichroic long pass filter (cut-on at 460 nm) was positioned in front of the slit to reject the back-scattered excitation light. The fluorescence spectrum was registered by the photodiode array (512 diodes) of an optical multichannel analyser (OMA) in the spectral range of 520–800 nm. The OMA could store up to 12 spectra before readout to a computer or a plotter. The background signal (noise and external light sources) was automatically subtracted from the fluorescence spectra.

Patients

Twenty persons of different age and sex were studied. Five of them suffered from the skin disease acne vulgaris.

Bacterial strain and growth conditions

Propionibacterium acnes was isolated from comedones and grown on Blood Agar Base no. 2, OXOID, pH 6.5, under dark conditions at 37 °C in anaerobic jars (Gas Pack System, OXOID) for 5 days. For the measurements the colonies grown on the agar were used. For calculation of the survival fraction after irradiation the colonies were removed from the agar plate, added to an anaerobic medium (RCM, OXOID) and diluted in steps of factor 10 down to 10^{-8}. Some 0.1 ml of the appropriate solution were spread on the surface of a B_2-agar plate and incubated in the anaerobic jar at 37 °C for 48 h prior to counting the grown colonies. Propionibacterium acnes, supplied from B. Kjeldstad (Trondheim, Norway), was used for comparative studies.

Chemicals

Protoporphyrin IX, coproporphyrin and Zn–protoporphyrin were obtained from Porphyrin Products (Utah, USA) and were used as supplied.

RESULTS

Measurements were performed on 20 human subjects aged between 16 and 45. All showed a strong autofluorescence in the red spectral region from the skin. Higher fluorescence intensities were obtained from patients with acne vulgaris than from persons without skin diseases. The highest fluorescence quantum yield was measured with 406.7 nm excitation (in comparison with the 356.4 nm and the 415.4 nm line of the krytpon ion laser) corresponding to the absorption maximum of the porphyrin monomers. The fluorescence mainly emitted from the face around the nose was found to arise from sebaceous follicles which contain large amounts of the skin bacterium Propionibacterium acnes.

The structured spectrum showed three main peaks around 600, 620 and 640 nm as well as of smaller shoulders in the long-wave red spectral region (Fig. 2). The intensity and the ratio of the fluorescence bands varied between different irradiated areas. In some cases even spectra with the 600 nm band but without peaks at 620 nm and 640 nm were found. The fluorescence could be clearly differentiated from the 'normal' autofluorescence of follicle-free skin areas (Fig. 2, lower curve). The spectrum of the fluorescent extrusions of some comedones as obtained by pressure extraction was comparable with the skin spectra. The cultures of Propionibacterium acnes isolated from the comedones and grown on agar plates produced fluorophores with a band around 635 nm, fluorophores with a band around 620 nm and fluorophores which emitted both bands.

In order to study the pH-dependence on the spectral distribution of the fluorescence, the pH value of the growth medium was varied in the range 5.0–6.9. The lowest fluorescence intensity was obtained for pH 6.9. The position of the spectral bands was independent of the pH-value in the range investigated.

The measured spectra of autofluorescence and the excitation maximum of about 400 nm are typical for porphyrins. In the fluorescence spectrum of protoporphyrin IX, dissolved in dimethylsulfoxide, a main emission peak appears at 632 nm; in the corresponding spectrum of coproporphyrin, the emission maximum is around 622 nm and Zn–protoporphyrin shows a maximum at 592 nm. It appears possible that the autofluorescence may be composed by different endogenous porphyrins.

Photobleaching experiments were carried out with bacterial cultures using the 407 nm radiation. The photo-induced changes of the spectra of the bacteria which produce protoporphyrin (635 nm species) are shown in Fig. 3. The intensity at 635 nm and 700 nm decreased, but the fluorescence intensity around 670 nm increased after application of the light doses indicated in Fig. 3. Photochemical reactions thus induce a photobleaching process as well as photoproduct formation. The fluorescence of the photoproduct at 670 nm reached a maximum and slowly decreased during further irradiation. In the case of bacteria producing only the coproporphyrin (emission maximum

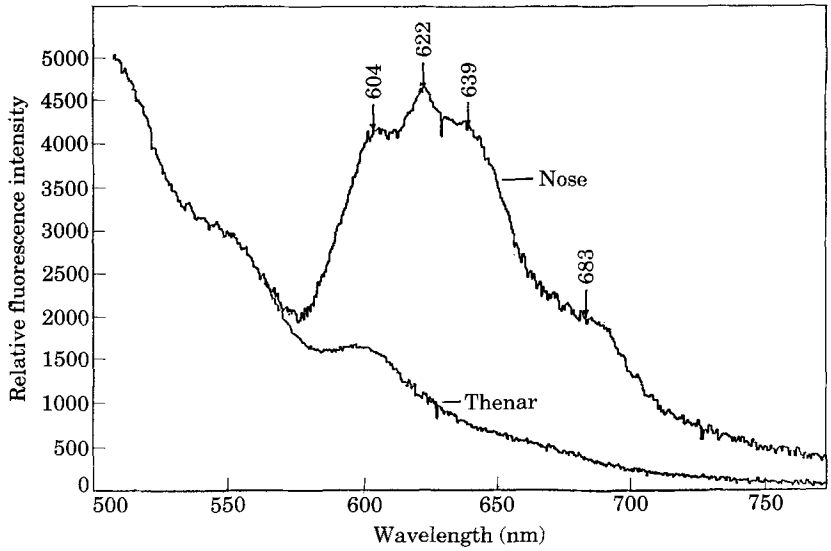

Fig. 2. In vivo spectrum of autofluorescence of the human skin around the nose (upper curve) and the thenar (follicle-free area, lower curve). Three main peaks occur around 600, 620 and 640 nm (λ_{EXC} = 407 nm, 5 mW cm^{-2}).

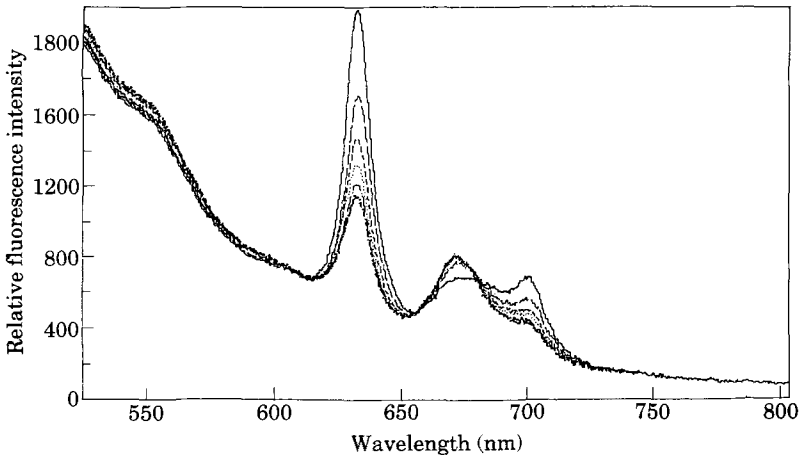

Fig. 3. Light-induced changes of the spectrum of bacterial colonies containing the 635 nm fluorophores during 407 nm irradiation with light doses of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 J cm^{-2} from upper to lower curve. Note the formation of fluorescent photoproducts at 670 nm.

at 620 nm), the fluorescence decreased upon irradiation, but no fluorescent photoproduct was observed.

The process of photobleaching was found to be oxygen dependent. In Fig. 4 the decrease of the fluorescence intensity during irradiation of comedones under normal (aerobic) conditions is depicted. With increasing light doses the 635 nm band dropped more rapidly in contrast to the 620 nm fluorescence band. Figure 5 (upper curves) shows the spectral behaviour under argon-flushed irradiation. The process of photobleaching is delayed. Stopping the oxygen reduction results in a fast decrease of the fluorescence intensity (lower curves).

Since porphyrins act as photosensitizers, the photo-induced cytotoxic effect on the bacteria was studied. Inactivation studies on bacterial colonies were carried out using the same excitation wavelength as for the fluorescence measurements (407 nm), but higher intensities (100 mW cm^{-2}). For the irradiation of the bacterial cultures aerobic conditions were used according to Kjeldstad (12, 16) who showed that singlet oxygen is involved in the inactivation process. During the irradiation the fluorescence spectra were recorded in real time. The light treatment was stopped when the integral fluorescence was diminished to 10% of the value before irradiation. A cytotoxic effect could be observed. Concomitantly the number of living bacteria was reduced by nearly a factor of 10, thus revealing the cytotoxic effect.

DISCUSSION

Endogenous porphyrins induce red auto-

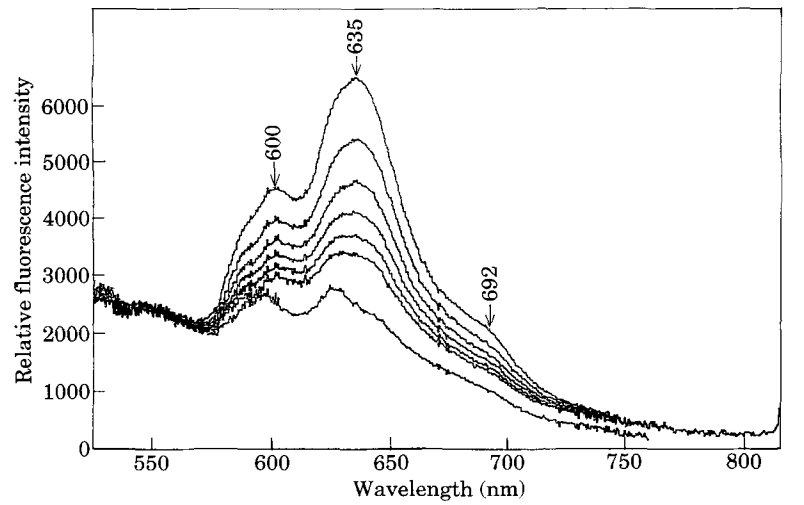

Fig. 4. Time-dependent fluorescence bleaching of comedones under normal (aerobic) conditions during irradiation (407 nm) with light doses of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 2.0 J cm^{-2} from upper to lower curve.

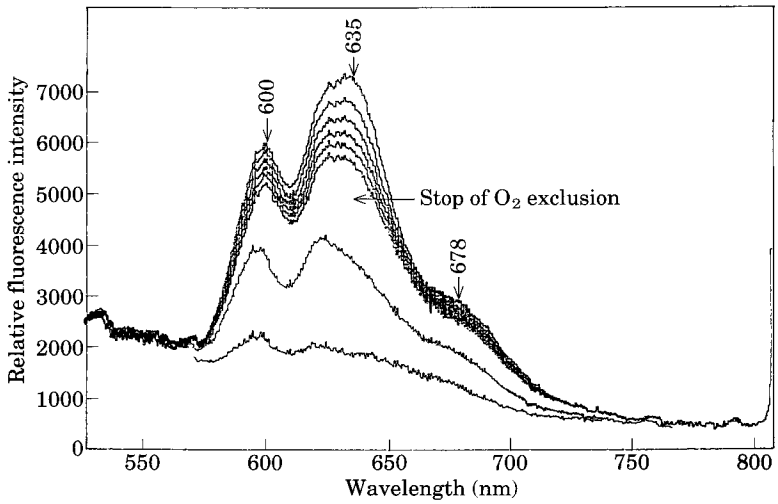

Fig. 5. Photobleaching under the condition of argon-flushed comedones (light doses: 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 J cm^{-2} from upper to lower curve). The lowest two curves were measured after stopping the anaerobic conditions and a dark phase of 2 min (light doses: 1.4 and 2.5 J cm^{-2}).

fluorescence of the human skin. The porphyrins are produced by the skin bacterium Propionibacterium acnes. The observed fluorescence is attributed to the existence of at least three fluorophores with different contributions in different skin areas. The positions and relative intensities of the fluorescence bands are characteristic of the metal-free porphyrins, protoporphyrin IX and coproporphyrin I, as well as of a fluorescent metalloporphyrin such as Zn–protoporphyrin.

A pH-dependence on the spectral behaviour of the bacteria in the pH range 5.0–6.9 (pH-value of the skin: 5.0–6.4 (4)) was not found. It is, however, possible, that the production of different porphyrins depends on the age and special growth conditions of the bacteria.

Irradiation of Propionibacterium acnes results in an oxygen-dependent photobleaching process. The fluorescence of protoporphyrin IX is reduced more efficiently as compared with the other endogenous porphyrins. Concomitant with a decrease in the integral fluorescence intensity, the formation of an additional band around 670 nm occurs which is due to the formation of photoproducts. As measured recently (17), the spectral bands and fluorescence decay times are typical for products induced by irradiation of protoporphyrin in lipophilic environments, the so-called photo-protoporphyrins.

The endogenous porphyrins may act as photosensitizers. Irradiation of Propionibacterium acnes, especially in the violet region, induces photo-oxidation processes which lead to phototoxic reactions as well as to the oxygen-dependent photodestruction of the sensitizer (photobleaching and formation of photoproducts). Therefore, a correlation between changes of the fluorescence intensity and the number of living bacteria exists.

According to this study, the use of endogenous porphyrins as photosensitizers for a photodynamic therapy of acne vulgaris seems to be possible. In addition, it may be possible to monitor the therapeutic effect of the light treatment by means of a simultaneous measurement of the spectral behaviour of the autofluorescence from the human skin.

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